TARGETING OF HER-2 OVEREXPRESSING BREAST CANCER CELLS WITH IMMUNOLIPOSOMES

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Abstract

The goal of the research described in this thesis is to develop a liposome based drug delivery system which targets Her-2 overexpressing breast tumors with high specificity. Overexpression of the Her-2 receptor occurs in many cancers but is most prevalent in breast tumors, with 20-30 percent of all cases displaying overexpression of the receptor. In addition, Her-2 overexpressing breast tumors are often aggressive and have a high probability of metastasizing. In the research reported here, a drug delivery system has been created that selectively targets Her-2 overexpressing mammary cells by combining three liposomal technologies. First, a Her-2 targeting antibody was conjugated to the outer surface of the liposomes, resulting in highly specific binding and internalization of liposomes into mammary epithelial cells that overexpress Her-2. Second, the liposomes were designed to be thermosensitive, only releasing their encapsulated cargo in response to mild hyperthermia at 42°C. Finally, the liposomes were attached to a pore-forming protein, listeriolysin O (LLO), which compromises the target cell endosome, allowing for drug delivery directly to the cellular cytoplasm. The liposomes delivered a 22-fold higher concentration of fluorescent marker to cells overexpressing Her-2 than to normal cells, demonstrating the delivery system's potential for targeting Her-2 overexpressing tumors. When a cytotoxin, gelonin, was encapsulated within the liposomes, the delivery system selectively targeted and killed Her-2 overexpressing cells *in vitro*. To further increase specificity for Her-2 overexpressing cells, the concept of a two-component delivery system was explored. This system would require internalization of two different types of liposomes within a cell endosome for effective drug delivery. Experiments using fluorescent markers show that this method greatly increased targeting specificity for Her-2 overexpressing cells.

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Chapter 1: Introduction

1.1 Challenges of Targeting Drug to Tumors

A drug delivery system targeting cancer cells must overcome several obstacles to attain therapeutic levels of chemotoxic agent within the tumor cells. Extracellularly, a delivery system must evade clearance by the immune system and be small enough to pass across the capillary endothelium (Werle and Berkop-Schnurch 2006, Kawai et al. 2009). Once across the endothelium, penetrating into the interstitial fluid to target tumor cells is a major problem for almost all drug delivery systems (Lunt et al. 2008, Igarashi 2008). For treatment of breast tumor cells that overexpress the human epidermal growth factor receptor 2 (Her-2), an optimal delivery system should selectively target the tumor region and also the Her-2 overexpressing cells within that tissue. Finally, for delivery of therapeutic agents that act intracellularly, the system must penetrate the target cell membrane and deliver drug directly into the cytoplasm of the cancer cells (Belting et al. 2005, El-Sayed et al. 2009). Overcoming these many obstacles requires a chemotherapy that can be modified and tailored to address each of the challenges. While the traditional chemotherapeutic approach of injecting a chemical into the blood stream leaves little room for modification, the rapidly emerging field of nanotechnology presents new opportunities for the design of drug delivery systems (Fenske et al. 2008). Polymeric nanovectors, polymeric micelles, dendrimers and lipid based nanoparticles are all nanotechnologies being developed for highly specific targeting of chemotherapeutic agents to cancer cells (reviewed by Amiji 2007).

1.2 Polymeric Nanovectors

Biodegradable polymers have been extensively studied as drug delivery nanoparticles, targeting malaria, HIV, diabetes, cancer and many other types of disease (Kumari et al. 2010). The polymeric nanoparticles are very stable compared to liposomes, showing

controlled release in the blood stream for up to several weeks (Kato et al. 1993, Jie et al. 2005). This stability coupled with a non toxic breakdown product has fueled large scale interest and resulted in a wide variety of polymeric particles that encapsulate many types of drug. The most common and well studied polymeric nanoparticles are composed of polylactic acid (PLA), polylactic acid co-glycolic acid (PLGA) or poly-ε-caprolactone (PLC) (Soppimath et al. 2001). Depending on the materials and conditions of formulations, these polymers form either nanocapsules or nanospheres (Soppimath et al. 2001, Kumari et al. 2010). Nanocapsules have a shell formed from the polymers with an aqueous compartment in which drug can be stored. Nanospheres consist of a polymer matrix that has pockets which can hold either water soluble drug or hydrophobic drug that is conjugated to the polymer. Release from the particles is generally through diffusion or degradation of the polymer, but pH sensitive and other controlled release strategies have been implemented into the technology (Hans and Lowman 2002).

The polymeric nanoparticle structure can be built up from monomers, but is most often created from preformed polymers (Amiji 2007). The solvent emulsion-evaporation method with preformed polymers involves dissolving the polymer in organic solvent, adding an aqueous solution along with a stabilizer, evaporating off the solvent and then sonicating to produce a homogenous polymeric nanoparticle solution (Soppimath et al. 2001, Kumari et al. 2010). Drugs can be absorbed onto the particle surface after production or more commonly can be added with the aqueous phase to the organic solvent resulting in a higher encapsulation efficiency. One drawback of polymeric nanoparticle systems has been that the acidic polymers can have degradative effects on the drug that is being encapsulated (Gao et al. 2005). However, recent methods of conjugating the polymers to other chemicals has resulted in more stable compounds that even allow efficient encapsulation and release of proteins.

1.3 Polymeric Micelles

Polymeric micelles are formed from amphiphilic block copolymers which spontaneously form a hydrophobic core with a hydrophilic corona in an aqueous environment (Gaucher et al. 2005). As with polymeric nanoparticles, polymeric micelles are generally formed from biodegradable polymers that show high stability and low immunogenic response *in vivo* (Kwon 1998, Kabanov et al. 2002). The hydrophobic segment is often PCL, PLGA or PGA while the hydrophilic section most often consists of polyethylene glycol (PEG), polyethylene oxide (PEO) or Poly vinyl pyrolidone (PVP). The micelles can be formed using the solvent emulsion-evaporation method but are more frequently just hydrated with an aqueous solution that contains the drug being encapsulated (Kabanov et al. 2002). The hydrophobic blocks of the polymer form the core where the hydrophobic drug accumulates. The particle size, pharmacokinetics, drug encapsulation efficiency and drug release characteristics are all highly affected by the type of polymer and the length of the hydrophobic and hydrophilic blocks (Nakanishi et al. 2001, Choucair and Eisenberg 2003). In general the hydrophilic block is larger than the hydrophobic block, which results in the formation of spherical micelles. However, when the hydrophobic block is larger, interesting morphologies including rods, vesicles and compound micelles can be formed (Choucair and Eisenberg 2003). Some of these structures are being utilized to co-encapsulate both hydrophobic and hydrophilic drugs, an elusive goal in drug delivery.

Polymeric micelles have shown success in delivering hydrophobic drugs such as doxorubicin and paclitaxel to tumor cells (Kwon 1998, Nakanishi et al. 2001). The micelles are very small, ranging in size from 10 to 100 nm, which helps them to avoid the immune system and to extravasate into the tumor tissue (Oku and Namba 1994). The micelles also seem to be effective in limiting the development of multi drug resistance (MDR), an effect that eventually renders small drug chemotherapies ineffective (Batrakova et al. 1999). The mechanism by which micelles interfere with MDR is not entirely understood, but seems to be a result of the micelles inhibiting drug efflux out of

the cell by transporter proteins, possibly through depletion of ATP production. There are currently at least four clinical trials being carried out with polymeric micelles (Amiji 2007). Although the variety of drugs that they can carry is limited, polymeric micelles show promise for increasing quantity of hydrophobic drugs at tumor sites.

1.4 Dendrimers

Perhaps the most complicated nanoparticle, dendrimers are polymers made from a particle core and grown layer by layer, polymers branching out until the reaction is terminated with peripheral functional groups (Hendrick et al. 2002, Gillies and Frechet 2005). The branches spread to the peripheral groups forming compartments in which a drug or molecule can be encapsulated (Figure 1.1). The most common polymers used for dendrimers are poly amidoamine (PAMAM) and poly L-lysine.

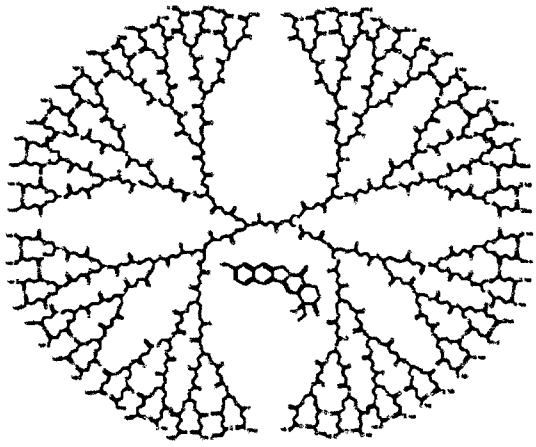

Figure 1.1. Example of a dendrimer structure

The structure of the dendrimer is extremely controlled with the size and chemical properties of the compartments being well characterized and adjustable depending on the chemical groups used for formation (Samad et al. 2009). Because of the difficulty in producing dendrimers, they are not as well studied as the other polymer and lipid based nanoparticles. However, their potential is well recognized. The tree like structures can

be made extremely small, ranging in sizes from just a few nanometers up to 12 nm with outer groups that can be modified and conjugated to antibodies and other targeting groups (Bosman et al. 1999). One of the most exciting uses for dendrimers is as a transfection reagent (Harada et al. 2000, Coles and Toth 1999). Cationic dendrimers can be associated with negatively charged DNA, resulting in the formation of DNA/dendrimer complexes. *In vivo* the positively charged dendrimers interact with the cell membrane, resulting in the complexes being internalized through endocytosis. The dendrimers have a high buffering capacity, causing swelling in the acidic endosomes and possibly rupturing the compartments, leading to delivery of the DNA to the cytoplasm (Boussif et al. 1995). This method for delivering DNA to the cells has advantages over traditional gene delivery methods that often involve immunogenic virus components.

1.5 Lipid Based Nanoparticles

Nanoemulsions, solid lipid nanoparticles (SLN), lipoprotein nanoparticles and liposomes are all lipid based nanotechnologies being developed using a mixture of lipids to produce nanometer sized drug carriers (Klang et al. 1998, Manjunath et al. 2005, Lacko et al. 2007). Nanoemulsions are dispersions of nanometer sized water droplets that contain pockets of oil and within the pockets of oil are hydrophobic drug molecules (Sarker 2005). The droplets are thermodynamically unstable and therefore require a surfactant molecule and a large amount of energy for their formation. Initially, the drug is mixed with the oil phase, which is made up of natural lipids, synthetic lipids, fatty acids or long chain triglycerides (Sarker 2005). The oil is mixed with water, a surfactant is added and the mixture is homogenized with a high pressure homogenizer or by sonication. The resulting nanoemulsion contains particles ranging in size from 1 to 100 nm in size that can be injected into the bloodstream for the delivery of hydrophobic molecules to cancer cells. One drawback of the nanoemulsion systems is that the required surfactant is often toxic and can limit the tolerable dosage of the nanoparticles (Klang et al. 1998).

Solid lipid nanoparticles are made up of a matrix of lipids that can include fatty acids, fatty esters, fatty alcohol and glycerides (Manjunath et al. 2005). The SLNs are formed by a variety of methods, but one of the more common techniques involves grinding the solid lipid into a fine powder under liquid nitrogen, dispersing the particles in a surfactant solution and homogenizing under high pressure (Trotta et al. 2003). As with nanoemulsions, the SLNs are most often used to encapsulate hydrophobic drugs, but modification of hydrophilic drugs with polymers can render the drug lipophilic, allowing for their encapsulation. SLNs can be surface modified to target certain cell types and increase their stability in the blood stream (Stevens et al 2004). One challenge of using SLNs is encapsulating large amounts of drug since the lipid matrix can be restrictive. The pores in the matrix do not hold the quantities of drug that some of the other nanoparticles are capable of storing. Despite this, SLNs have shown potential to deliver hydrophobic drugs which interfere with transporters that pump drug out of the cells such as P-glycoprotein. This interference allows for increased sensitivity to chemotherapies such as doxorubicin and paclitaxel (Ma et al. 2009).

Lipoprotein are naturally occurring complexes, consisting of phospholipids, apolipoproteins and cholesterol that serve the purpose of transporting water-insoluble lipids throughout the body (Lacko et al. 2007). Plasma lipoproteins form particles that are 5 to 1000 nm in size. The main advantages of using lipoproteins for drug delivery are that the particles are biologically stable and that cells express receptors that bind and endocytose the particles (Williams et al. 2000). These lipoprotein receptors are often overexpressed by malignant cancer cells and represent a way to specifically target drug to the cancer (Imachi et al. 1999). Although, the potential of lipoprotein particles is apparent, study has been restricted due to the difficulty of obtaining adequate starting materials. The lipoprotein components can be difficult to purify and in particular the lack of apolipoprotein, a low density lipoprotein, has limited advancements in this nanotechnology (Lacko et al. 2007).

Liposomes are arguably the best characterized and most successful nanoparticle used for cancer treatment (Torchilin and Weissig 2003). Liposomes have been extensively studied since their development in 1965 with over 20,000 papers in the literature discussing these nanoparticles (Bangham et al. 1965). There are at least 3 FDA approved liposome based cancer therapies and 25 current clinical trials involving targeting tumors using liposomes (Amiji 2007). Given their ease of production and potential for delivery, liposomes were chosen as the nanoparticle to study in this doctoral project.

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Chapter 2: Liposomes for Tumor Targeting

2.1 Liposome Structure and Formation

Liposomes consist of a lipid bilayer, formed spontaneously when amphiphilic lipids are introduced into an aqueous solution (Lasic and Papahadjopoulos 1998, Tran et al. 2009). The hydrophobic tails of the lipids cluster together with the hydrophilic head groups facing outwards, forming an aqueous interior, much like a small cell. Hydrophilic drugs can be encapsulated in large quantities within the liposome interior while hydrophobic drugs can be placed within the lipid bilayer. Liposomes are classified into three groups; multilamellar liposomes, large unilamellar liposomes (LUV) and small unilamellar liposomes (SUV). Multilamellar liposomes and LUVs are larger in size, between 500 nm and 1 micron (Torchilin and Weissig 2003). The multilamellar liposomes contain many overlapping layers of liposomes while the LUVs and SUVs contain a single bilayer surrounding the aqueous interior. SUVs, usually in the 100 nm size range, are the liposomes studied most intensely for cancer therapy and are the liposomes used for all of the FDA approved cancer therapies (Amiji 2007).

The two techniques typically used for the formation of liposomes are the film hydration method and the reverse phase evaporation method (Torchilin and Weissig 2003). The film hydration method is the most simple and involves drying the lipids onto the side of a glass test tube, and rehydrating with a buffer solution that contains the drug to be encapsulated. The rehydration of the dried lipid forms multilamellar liposomes which can be subsequently formed into LUVs or SUVs by sonicating or filtering. The reverse phase evaporation method requires dissolving lipids in chloroform and then mixing them in a methanol/chloroform/buffer solution which contains the drug dissolved in the buffer. The organic solvents are slowly evaporated until the lipids become unstable and spontaneously form LUVs in the aqueous solution (Otake et al. 2006). This method has

been shown to encapsulate more drug than the film hydration method since the vesicles form a unilamellar bilayer without passing through a multilamellar confirmation.

After their invention, liposomes were immediately recognized for their possible drug delivery uses. The vesicles offered several advantages over traditional chemotherapies. The lipid bilayer of the liposome protected large quantities of encapsulated drug, allowing for the possibility of prolonged circulation and delivery. Liposomes had the advantage of restricting undesired drug delivery to certain normal tissues, such as reducing the exposure of chemotherapeutic cardiotoxic anthracyclines to heart tissue (Gabizon 2001). Finally, liposomes could be functionalized and modified so that they might target certain tumor tissue and even tumor cells (Park et al. 2001, Wartlick et al. 2004). While the advantages of liposomes may have been immediately recognized, they were not quickly realized. The many obstacles to effective drug delivery have prevented all but three liposome designs from reaching the market even after 45 years of research (Igarashi 2008). Each of these obstacles has been met with innovative designs from the past, and current strategies being developed today. The first and perhaps most difficult obstacle which required 25 years to overcome was to avoid clearance of liposomes by the immune system (Allen and Chonn 1987, Gabizon and Paphadjopoulos 1988).

2.2 Clearance of Liposomes by the Immune System

Liposomes injected intravenously are rapidly cleared from circulation by the reticuloendothelial system (RES) (Immordino et al. 2006). The RES consists of monocytes and macrophages which phagocytose liposomes, leading to their clearance by the liver, spleen and lungs. In the early progress of the liposome field, it was noted that after administration the liposome half-life in a mouse was less than 30 minutes, preventing adequate drug delivery to tumor tissue (Klibanov et al. 1990). While the liposomes were large enough to avoid glomerular filtration by the kidneys, a method for

avoiding clearance by the RES system was necessary for the possibility of effective drug delivery.

Modification of surface charge and cholesterol concentrations marginally improved liposome half-life, but it was not until 1987 that Allen and Chonn showed how inclusion of ganglioside and sphingomyelin in the lipid bilayer drastically improved liposome circulation time (Allen and Chonn 1987, Gabizon and Paphadjopoulos 1988). Another leap was made in 1990 when Klibanov *et al.* demonstrated that a polyethylene glycol (PEG) coating resulted in a liposome that was even more effective at avoiding clearance by the immune system (Klibanov et al. 1990). PEG incorporated into liposomes increases liposome circulation by at least 5-fold with some formulations showing a half-life of up to 20 hours (Papahadjopoulos et al. 1991). These liposomes coated with PEG, ganglioside and sphingomyelin, known as "stealth liposomes", are used today in most *in-vivo* liposome studies.

Clearance of liposomes is thought to be the result of opsonization by serum proteins, including immunoglobulin, fibronectin and complement proteins (Patel 1992, Immordino 2006). In particular, the complement system is thought to play a large role in liposome clearance, initially forming pores in the liposome and then aiding in their phagocytosis. The predominate theory for how PEG increases circulation time is that the large polymers sterically restrict complement and other proteins from binding to the surface of the liposomes (Drummond et al. 1999). Other reports argue that proteins still bind to the liposome but that the PEG restricts binding of the phagocytotic cells to the surface bound opsonins (Johnstone et al. 2001). Regardless of the mechanism, the discovery of stealth liposomes spurred development of liposome technology, resulting in liposomes being the most studied nanoparticle for cancer delivery. However, recent reports are beginning to indicate that the immune system may not have been so easily overcome.

Two groups have reported that while PEG can increase the half-life of the first dose of liposomes injected intravenously, the following injected dose is met with a heightened immune response, resulting in rapid clearance of the liposomes (Laverman et al. 2001, Ishida et al. 2005). The multiple injection tests have been performed on monkeys, mice and rats all with the same results, a reduced liposome circulation time with injections subsequent to the primary dose. It has been shown that this heightened sensitivity is due to the production of IgM antibody which targets the PEG, resulting in lipsosome uptake (Ishida et al. 2005). The response takes about 4 to 7 days to develop but then lasts for 5 weeks or possibly longer. The flaw of earlier studies on stealth liposomes is that they did not generally perform a second injection and therefore did not reveal this effect called the accelerated blood clearance (ABC) phenomenon. The ABC effect is cause for great concern since it essentially negates the development of stealth liposomes and brings the technology back to an obstacle thought to be overcome in the 1980's. However, recent experiments are showing that there may be strategies for overcoming the ABC effect. When the primary injection of liposomes is very large or the liposomes contain a toxic chemotherapeutic agent, the ABC effect is ablated (Laverman et al. 2001, Ishida et al 2006). This is thought to be due to harmful levels of lipid or damage from the toxic agent, reducing the activity of the B cells and phagoctyotic cells, thereby limiting the immune response. Studies continue on this subject and will hopefully reveal strategies that are effective in retaining the protective qualities of PEG coated stealth liposomes.

2.3 Extravasation of Liposomes past the Capillary Endothelium

Tumors that have grown past 1 cm^3 in size must develop a vasculature system if they are to continue growing (Weinberg 2007). These rapidly grown vascular beds are aberrant in their design, displaying several characteristics that are not found in normal blood vessels (Torchilin 2000, Maeda et al. 2009). The tumor vessels have a defective architecture with openings between endothelial cells that range from 10 nm to 500 nm. The vessels lack a smooth muscle layer, are often in high density and have low venous return rates.

All of these characteristics aid in the targeting of tumors using liposomes through an effect called enhanced penetration and retention (EPR).

A healthy capillary endothelium found in skeletal muscle and skin is continuous, containing tight junctions, and allows only diffusion and receptor mediated transport to the interstitial fluid. Some organs such as the intestines, pancreas and kidneys are fenestrated with pores between the endothelial cells that are 60-80 nm in diameter (Pavelka and Jürgen 2005). In general, conventional chemotherapeutic agents diffuse across the cellular membrane and the capillary endothelium, targeting most bodily tissues within ten minutes of injection (Noguchi et al. 1998). For these drugs, there is little difference in the concentration of drug in tumor and normal tissue. However, liposomes do not diffuse freely past endothelial cells, requiring either capillary junctions or transport to reach the interstitial fluid. Because of the leaky vasculature in tumor tissue, liposomes that are under 500 nm accumulate more rapidly in the tissue interstitium. After 6 hours of circulation, this enhanced penetration effect can lead to concentrations of liposomes that are 30-300 greater in tumor tissue than in normal tissue (Maeda et al. 2009). In addition, tumors are characterized by poor lymph drainage. Normal tissue with a leaky vasculature will clear liposomes through lymph drainage within a few days, while in tumors the liposomes can remain for several weeks (Konno et al. 1983, Maeda et al. 2009). It is this EPR effect which allows a passive liposome accumulation in tumor tissue and forms the basis of many liposome therapies. The most common targeting strategy is to fill liposomes with small chemotherapeutic agents, which diffuse slowly from tumor localized liposomes (Gabizon 2001).

An interesting effect of the rapid blood vessel development in tumors is the lack of smooth muscle cells lining the endothelial cells (Nagamitsu et al. 2007). Researchers have taken advantage of this effect by introducing angiotensin-2 into the blood, which causes normal endothelium to vasoconstrict, resulting in an increase in blood pressure. The reduced diameter of normal vessels limits the blood flowing through the normal

tissue. However, the tumor vessels lack the smooth muscle cells and are therefore unable to vasoconstrict, resulting in an increased blood flow which can enhance liposome accumulation by up to 3-fold (Li et al. 1993).

2.4 Liposomes Penetrating into the Tumor Interstitium

The tumor interstitial environment provides a formidable barrier for liposome based drug delivery systems (Lunt et al. 2008). If a drug delivery system is simply trying to release drug into the vicinity of the tumor cells, then extravasation into the tumor perimeter may be adequate for drug delivery. However, if a liposome delivery system functions by targeting drug directly to the tumor cell cytoplasm, the liposomes must come into contact with the tumor cells. This is a challenge given the typical tumor interstitium. Although, most tumors exhibit hypervascular regions with rapidly growing cells, there are also regions with poor blood delivery containing quiescent or necrotic cells (Weinberg 2007). Unless tumor cells are directly adjacent to a leaky capillary junction, there may be little exposure to liposomes. Studies show that a typical small drug can penetrate into the interstitium up to 200 um while the more bulky 100 nm liposomes seem to penetrate only to 10 um, well short of cells deep within a tumor (Chen et al. 2008). Experiments with 40 nm and 100 nm colloids show that the 100 nm colloids diffuse through the interstitial fluid at about half the rate of the 40 nm colloids (Kawai et al. 2009). There are several factors that prevent a quicker penetration. The first is simply mechanical barriers. The cells within the tumor tissue are thought to be separated by about 100 nm, the width of one liposome, and this space is filled with fibronectin, integrin and other components of the extracellular matrix (Kawai et al. 2009). The other problem is that tumors generally have a high interstitial pressure (Nathanson and Nelson 1994). One study showed that the pressure in normal tissues was -3.0 mm Hg while the breast tumor had pressures of 29 mm Hg. This high tumor pressure which is a result of poor lymphatic drainage creates a pressure gradient that moves macromolecules away from the tumor center.

Solutions are limited for a more effective targeting of tumor cells deep within the interstitial fluid. One group has tried to achieve deeper penetration by attaching nanometer magnetic particles to the lipid bilayer of liposomes and guiding the magnetic liposome into the tumor depths with a strong magnetic field gradient (Martina et al. 2007). While this has shown some success, the procedure may be difficult to reproduce in a human patient with cancer. The study was performed on mice that had tumors bulging from the skin with a strong magnet placed directly against the tumor. A breast or prostate tumor located within the tissue would be in a difficult location to achieve such a high magnetic field gradient. The magnetic particles also lead to liposome aggregation, higher clearance rates and possibly higher toxicity.

One exciting approach to increasing liposome penetration involves the use of small arginine-glycine-aspartic acid (RGD) peptides that bind to the integrin components of blood vessels (Sugahara et al. 2009, Teesalu et al. 2009). Integrins constitute one of the most diverse family of proteins in the genome with 18 alpha-subunits and 8 beta-subunits. The variation of integrin and other components in the vasculature of different tissues creates what has been described as a vasculature zip code. By testing a library of RGD peptides, Ruoslahte's lab was able to isolate peptides that bind specifically to a tumor vasculature (Teesalu et al. 2009). Their most recently isolated RGD peptides bind to the integrin component of the blood vessel where proteolytic cleavage creates a conformation that binds to neuropilin-1, resulting in internalization into the endothelial cell. Interestingly, after being internalized, the RGD peptides did not simply collect in the endothelial cells, but were transported through the vasculature and deep within the tumor interstitial fluid. When these RGD peptides were attached to nanoparticles, those particles also bound to the tumor vasculature system and were similarly transported deep into the tumor's poorly vascularized regions (Teesalu et al. 2009). Whether the mechanism of transport is trancytosis through the cells or some other form of movement

along the extracellular matrix, this result provides a possible solution to the dilemma of how to target drug to all of the cancer cells within a tumor.

2.5 Targeting Tumor Cells that Overexpress Her-2

Overexpression of the human epidermal growth factor receptor 2 (Her-2) occurs in 20-35 percent of breast cancers and typically corresponds with an aggressive and metastatic tumor (Engel and Kaklamani 2007, Azambuja et al. 2008). Her-2 is located in the cell plasma membrane and can be easily targeted with commercially available antibodies, making the receptor a logical target for chemotherapies (Johnston et al. 2006). After antibodies bind to Her-2, antibody-receptor complexes are clustered and internalized into cell endosomes, providing an opportunity for selective drug delivery into the cell cytoplasm (Park et al. 2001, Wartlick et al. 2004, Yang et al. 2007).

Because Her-2 offers such a clear target in breast cancer, many chemotherapies have been designed to take advantage of the receptor overexpression. The simplest of the Her-2 targeting therapies uses trastuzumab, which is an antibody that binds Her-2 on the cell plasma membrane (Valabrega et al. 2007). The mechanism through which trastuzumab decreases cancer cell proliferation is not entirely clear, but it most likely involves interference of Her-2 function combined with an increase in immune response (Albanell et al. 2003). One shortcoming of using trastuzumab alone is that it does not actively kill the Her-2 overexpressing cancer cells and eventually most tumors become resistant to the therapy (Tolaney and Krop 2009). Another treatment conjugates a chemotherapeutic agent directly to a Her-2 targeting antibody, resulting in internalization of the chemotherapeutic agent into the cancer cell's endosomes (Zhang et al. 2008, von Minckwitz et al. 2005). There are several problems with this technique. The chemotherapeutic agents are cleared quickly from the blood stream by the reticuloendothelial system, and drug that does reach the target cell is often trapped within the cellular endosomes. Another problem is that the chemical conjugation of a single

drug molecule to an antibody restricts the quantity of drug that can be delivered to the tumor cells (Woodhams et al. 2009). Perhaps the most promising approach used for targeting Her-2 overexpressing cells involves the conjugation of antibody to a liposome.

Her-2 targeting liposomes were created in 1995, consisting of liposomes conjugated to an anti-Her-2 antibody (Park et al. 1995, Suzuki et al. 1995). Park *et al.* demonstrated that the liposome filled with doxorubicin effectively targeted and treated Her-2 overexpressing tumors in mice. Soon after their development, PEG was conjugated to the targeted liposomes, but internalization and drug delivery seemed to be impaired by the long polymers (Goren et al. 1996). Again, Park's lab was at the forefront of the technology, demonstrating that antibody had to be conjugated to the distal end of the PEG molecule to preserve the conjugation and internalization of liposomes into Her-2 overexpressing cells (Kirpotin et al. 1997). Since then, Her-2 targeting liposomes have been studied by a number of groups delivering anti-cancer agents to breast cancer cells that overexpress Her-2 (Gao et al. 2009, Kikumori et al. 2008). To date, there is no literature on treating Her-2 overexpressing tumors in humans. Park's lab recently studied the accumulation of Her-2 targeted liposomes versus non-targeted liposomes in mice tumors that overexpress Her-2 (Kirpotin et al. 2006). They showed that the EPR effect results in similar levels of liposome accumulation, but that the Her-2 targeted liposomes bound and internalized into the cancer cells while the non-targeted liposomes were found in extracellular regions and macrophages. The enhanced effectiveness of Her-2 targeted liposomes is directly related to their internalization. This study is the first and perhaps only one to show receptor mediated internalization of antibody targeted liposomes *in vivo*.

2.6 Cytoplasmic Delivery to Tumor Cells using Liposomes

A growing number of therapeutic macromolecules, including small RNAs, DNAs, and proteins act intracellularly, but have no independent mechanism for penetrating the

cellular membrane (El-Sayed et al. 2009). For cancer therapies to progress past small membrane permeable compounds, we must develop efficient methods for achieving cytoplasmic delivery of macromolecules. Liposomes offer a promising solution since they can encapsulate and protect large quantities of the macromolecules and with the right targeting molecules can deliver the macromolecules to the endosomes of cells. However, enabling the escape of therapeutic molecules from the endosomal compartments before lysosomal degradative enzymes render the molecules ineffective remains an intense topic of research (Xu et al. 2008, El-Sayed 2009). There are several methods that exist for increasing cytoplasmic delivery from endosomes.

The introduction of pH sensitive lipids into the lipid bilayer of liposomes can increase cytoplasmic delivery from endocytosed liposomes (Torchilin and Weissig 2003). Upon acidification of the endosome, the pH sensitive lipids go through a phase transition that destabilizes the lipid bilayer and promotes the fusion of the liposome and endosome. The fusion releases the encapsulated drug into the cellular cytoplasm. Several difficulties remain with the pH-sensitive approach. One difficulty is that attaching PEG to the pH-sensitive liposomes results in a physical barrier between the endosome and liposome that almost completely prevents membrane fusion (Momekova et al. 2010). There has been much study, with some success, on creating PEG molecules that dissociate within the endosome so that the liposome and endosome membranes can come into contact (Xu et al. 2008). Other methods for increasing the fusion between liposome and endosome involve the conjugation of viral or toxin components that undergo conformational changes at low pH which allow the liposome to fuse with the membrane (Kakimoto et al. 2009, El-Sayed et al. 2009). These have not been as extensively studied as pH lipids but have been reported to increase cytoplasmic delivery of macromolecules. Since these compounds are directly conjugated to the lipid bilayer their effectiveness will most likely also be restricted by the addition of PEG to the liposomes.

Although cell penetrating peptides (CPPs) have received much attention in the last decade as a possible method for targeting the cytoplasm, their ability to penetrate the endosomal barrier appears limited (El-Sayed et al. 2009). When small molecules are conjugated to CPPs they are delivered to the cytoplasm of all cells. The proposed method of delivery is still controversial, but generally believed to be the result of the CPPs being internalized through the endocytotic pathways (Suzuki et al. 2002, Richard et al. 2003). At first it was thought that CPPs had a way of escaping the endosomal compartments but, with regard to macromolecules and liposomes, CPPs do not seem to allow for delivery to the cytoplasm (El-Sayed et al. 2009).

The method of photochemical internalization (PCI) has been shown to increase the cytoplasmic delivery from internalized liposomes (Fretz et al. 2007, Bøe et al. 2007). This technique involves the addition of a photosensitizer to the tissue. The photosensitizer is internalized into cellular endosomes and upon illumination with a light source, the photosensitizer produces highly reactive singlet oxygen species. These reactive singlet oxygen species break down the endosome, facilitating the release of any endosomal macromolecules into the cellular cytoplasm. One difficulty with this approach is that the light source does not penetrate deeply through the tissue and limits the success of the therapy to tumors near the skin surface (Hogset et al. 2004, El-sayed 2009). Also, the activation of the photosensitizer can cause significant damage to the therapeutic macromolecule that is in the endosome. There is a delicate balance between having enough photosensitizer to breakdown the endosome but not so much as to reduce the efficacy of the therapeutic macromolecule.

A promising approach for cytoplasmic targeting utilizes a pore forming protein, Listeriolysin O (LLO), which is produced by the bacterium *Listeria monocytogenes* (Schnupf and Portnoy 2007). When the bacterium infects a cell and is internalized into a cellular endosome, it releases LLO, which forms pores in the endosome membrane, allowing the bacterium access to the cellular cytoplasm. Lee *et al.* co-encapsulated purified LLO and a therapeutic protein into pH sensitive liposomes and showed that

when LLO was released into the endosomal compartment it formed pores in the endosomal membrane (Lee et al. 1996, Provoda et al. 2003). The therapeutic protein flowed through these pores into the cytoplasm where it could effectively treat the cell. This method has been studied only by Lee *et al.* but is a creative approach that offers an alternative to the standard method of promoting liposome fusion with pH-sensitive components.

2.7 Doctoral Research Project

The doctoral work described in this manuscript was focused on developing and advancing the field of Her-2 targeting liposomes. Her-2 overexpressing tumors are often highly metastatic cancers that are associated with a low patient survival rate. To better treat these aggresive tumors we must have more effective chemotherapies. Liposomes that are conjugated to Her-2 targeting antibody have the potential to deliver drug with high specificity to Her-2 overexpressing tumors. The first aim of our work was to develop a thermosensitive liposome that could target Her-2 overexpressing cancer cells *in vitro*. Chapter 3, a manuscript published in the *Journal of Drug Targeting* (Kullberg et al. 2009), describes a technique for conjugating the Her-2 specific antibody trastuzumab to stealth liposomes using chemistry that results in the antibody being attached to the distal end of the PEG molecules. Using these liposomes, several targeting strategies *in vitro* were tested with the hopes of increasing specificity for Her-2 overexpressing cells.

After successfully delivering a fluorescent marker to the endosomes of Her-2 overexpressing cells, our next aim was to penetrate the endosomal membrane and deliver the marker directly to the cellular cytoplasm. Many of the recently developed chemotherapeutic agents are marcromolecules or nucleic acids, whose activity is dependent on them reaching the cytoplasm. It is vital that effective techniques be developed for getting these chemotherapeutic agents past the cellular membrane and into the cellular cytoplasm. Chapter 4 of this thesis is a manuscript in press at the *Journal of*

Drug Targeting, describing a method for increasing cytoplasmic delivery by Her-2 targeted liposomes using the pore forming protein LLO. The approach is similar to that of Lee *et al.* but instead of encapsulating LLO within pH-sensitive liposomes, the LLO is attached directly to the lipid bilayer of Her-2 targeting heat-sensitive liposomes (Lee et al. 1996). Results of experiments in which we have encapsulated a cytotoxin within the liposomes and tested the ability of the liposomal delivery system to selectively kill Her-2 overexpressing cells *in vitro* are reported in chapter 5.

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Chapter 3: A Two-Component Drug Delivery System using Her-2 Targeting Thermosensitive Liposomes[1]

3.1 Abstract

We report on a new method for enhancing the specificity of drug delivery for tumor cells, using thermosensitive immunoliposomes. The liposomes are conjugated to the antibody trastuzumab (Herceptin®), which targets the human epidermal growth factor receptor 2 (Her-2), a cell membrane receptor overexpressed in many human cancers. Being thermosensitive, the liposomes only release their contents when heated slightly above body temperature, allowing for the possibility of tissue targeting through localized hyperthermia. Using self quenching calcein, we demonstrate release of liposome contents into cell endosomes after brief heating to 42°C. To further increase targeting specificity, we incorporate the concept of a two-component delivery system that requires the interaction of two different liposomes within the same endosome for cytoplasmic delivery. Experimental evaluation of the technique using fluorescently labeled liposomes shows that a two-component delivery system, combined with intracellular disruption of liposomes by hyperthermia, significantly increases specificity for Her-2 overexpressing tumor cells.

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3.2 Introduction

Overexpression of the human epidermal growth factor receptor 2, Her-2, occurs in 20-35 percent of breast cancers and is associated with an aggressive and lethal phenotype (Engel and Kaklamani 2007, Azambuja et al. 2008). Some level of Her-2 overexpression is documented in many other types of cancer, including prostate, brain, bladder and lung tumors (Canoz et al. 2006, Nishio et al. 2006, Haynik et al. 2007, Jalali Nadoushan et al. 2007) This common upregulation makes Her-2 an attractive target for drug delivery. In addition, the receptor is located in the cell membrane and can be easily targeted with commercially available antibodies (Johnston et al. 2006, Friedländer et al. 2008). After binding to Her-2, the antibody-receptor complexes are clustered and internalized into cell endosomes, providing an opportunity for selective drug delivery into the cell cytoplasm (Park et al. 2001, Wartlick et al. 2004, Yang et al. 2007). In this paper, we describe a technique for targeting liposomes to cancers that overexpress Her-2. This liposome-based delivery system improves targeting of tumor cells by combining three levels of specificity:

1. **Antibody Targeting:** The liposomes are conjugated to the antibody trastuzumab (Herceptin®), which targets Her-2. This antibody allows the liposomes to attach with great specificity to the surface of Her-2 expressing cells. Once bound to the cells, the liposome-receptor complexes are engulfed into endosomal compartments.

2. **Thermosensitive Liposomes:** The liposomes are thermosensitive, only releasing their contents when heated slightly above body temperature (Needham et al. 2000). If this strategy is implemented as a therapy, a localized thermal source would heat the region of the tumor to 42°C, causing a release of encapsulated compounds from the liposomes in the tumor tissue (Kakinuma et al. 1996, Hauck

et al. 2006). Liposomes that have bound to Her-2 and been internalized should release their contents inside the endosomes of cells exposed to the elevated temperature.

3. **Two-Component Strategy:** Specificity is further amplified by using a two-component strategy that preferentially increases the likelihood of effective drug delivery to Her-2 overexpressing cells (Kullberg et al. 2005).

3.2.1 *Theoretical Basis of a Two-Component Delivery System*

The specificity of drug delivery can possibly be enhanced by using a delivery strategy that requires two separate components to interact within an endosome of the target cell before drug is delivered. The components are two sets of Her-2 targeting thermosensitive liposomes that differ only in the substance they encapsulate. After the liposomes bind to Her-2, the liposome-receptor complexes are engulfed into endosomes, which travel through the endocytotic pathway (Park et al. 2001, Yang et al. 2007). Endosomes fuse together to create larger vescicles which fuse with late endosomes and lysosomes. As endosomes invaginate from the cell surface and fuse, there is a probability that two liposomes, each containing a different substance, will colocalize into one endosome. Localized hyperthermia would cause the thermosensitive liposomes to release the two substances into the endosome where they could work together to deliver drug into the cell cytoplasm. The upregulation of receptor on a tumor cell surface causes a proportional increase in the number of receptors that are internalized into each endosome (Benveniste et al. 1989). The colocalization of two Her-2 bound liposomes inside the same endosome is therefore more probable in a Her-2 overexpressing tumor cell than in a normal cell (Figure 3.1). Because two types of liposome must overlap within the same compartment for drug to be released into the cytoplasm, tumor cells are statistically likely to receive more drug than normal cells.

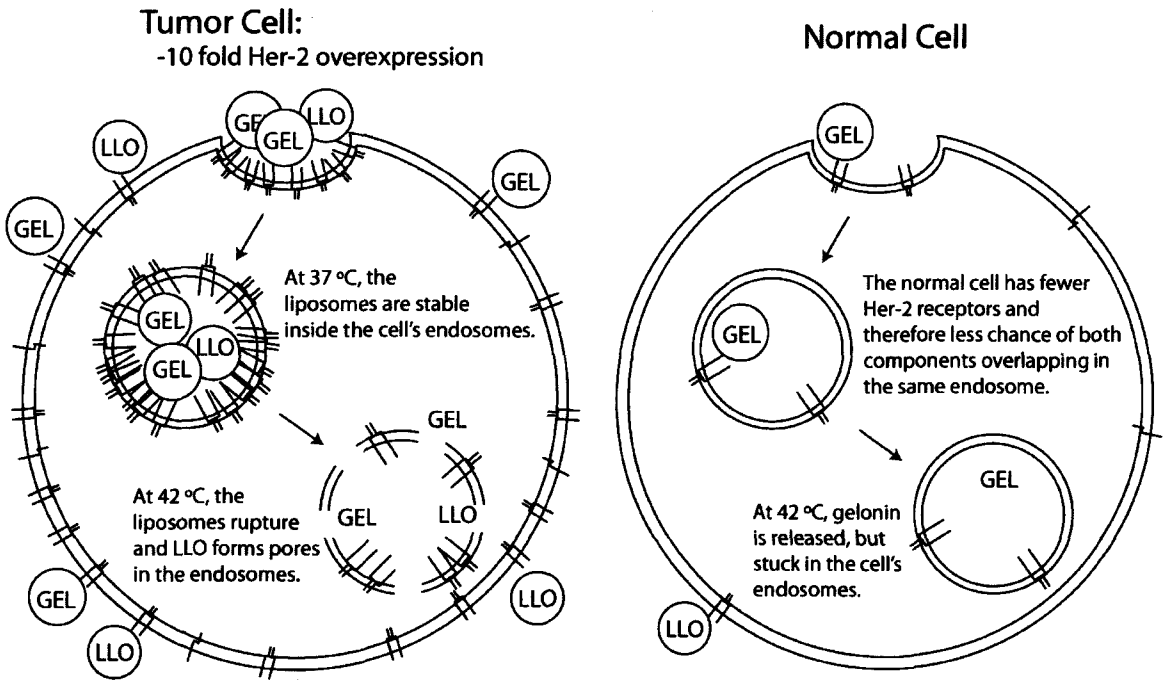


Figure 3.1. Theoretical two-component delivery system. The two-component system would require the overlap of two liposomes inside of a cell endosome before delivering drug to the cell. In this illustration, the two-components are LLO and gelonin (GEL). After heat triggered release of the thermosensitive liposomes, LLO forms pores in the endosome membrane that allow gelonin access into the cytoplasm. Gelonin cannot escape an endosome on its own, but is extremely toxic once it reaches the cytoplasm.

The substances that are encapsulated in the liposomes must be chosen so that their interaction results in active drug escaping from the endosome and being delivered into the cell cytoplasm. Two candidates for a dual component therapy are the pore-forming protein Listeriolysin O (LLO) and the toxin gelonin (Provoda et al. 2003). Provoda et al. coencapsulated LLO and gelonin into a single set of pH-sensitive liposomes and allowed cells to engulf the liposomes. When the LLO and gelonin colocalized in a single endosome, LLO utilized cholesterol to form pores in the endosome. Gelonin traveled through these pores into the cytoplasm where it inhibited ribosome activity, resulting in cell death. If either substance was encapsulated by itself, there was minimal cytotoxicity. Gelonin on its own could not escape the endosome to harm the cell, and LLO on its own

caused no significant cellular damage. It should be possible to create a targeted two-component therapy by separating the LLO and gelonin and encapsulating them into two different sets of Her-2 targeting thermosensitive liposomes. After the liposomes bind to Her-2 and are internalized into the cell, it is expected that localized hyperthermia of the tumor tissue will release the liposome contents into the endosomes. If released together into a single endosome, LLO and gelonin will work together to kill the cell.

To evaluate the feasibility of using a two-component system for targeted drug delivery, we observed the internalization of two sets of thermosensitive liposomes containing either a fluorescent rhodamine probe or a fluorescent calcein probe. The two types of fluorescent liposomes served as a model for the two sets of liposomes that will eventually make up the therapy. By analyzing overlap of the probes in the endosomes of a mammary epithelial cell line engineered to overexpress Her-2 by 10-fold, we tested the effectiveness of a two-component delivery system.

3.3 Materials and Methods

3.3.1 Materials

1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[poly(ethylene glycol)-3400]-N-hydroxy succinamide (DSPE-PEG(3400)-NHS) was purchased from Shearwater Polymers (Huntsville, USA), now Nektar Therapeutics (San Carlos, USA). The fluorescent probe, Lissamine™ rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (RhoPE) was purchased from Molecular Probes (Eugene, USA). 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (DPPC), 1-Palmitoyl-2-Hydroxy-sn-Glycero-3-Phosphocholine (MPPC), 1,2-Dipalmitoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (DPPG) and 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] (DSPE-PEG(2000)) were purchased from Avanti Polar Lipids (Alabaster, USA). CL-4B Sepharose gel, used for purification of the liposomes, was purchased from Amersham Biosciences (Uppsala, Sweden).

The cell lines MTSV1-7 and ce2 were kindly supplied by Dr. Joyce Taylor-Papadimitriou at the Breast Cancer Biology Group, King's College London School of Medicine, U.K. Trastuzumab was a generous gift from Dr. Max Rabinowitz at Alaska Oncology and Hematology, Anchorage, AK. Calcein and all other chemicals and reagents were purchased from Sigma Chemicals Company (USA).

3.3.2 *Cell culture*

The human mammary epithelial cell lines MTSV1-7 and ce2 were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1 μM insulin, and 5 μM dexamethasone and kept at 37°C in an atmosphere of 5% CO_2. Ce2 cells were derived from MTSV1-7 cells that had been stably transfected with Her-2 DNA (D'Souza et al. 1993, Worthylake et al. 1999). G418 was added to the medium at a concentration of 250 μg/ml to maintain Her-2 expression.

3.3.3 *Preparation of liposomes*

All liposomes were prepared by the film hydration-extrusion method (Torchilin and Weissig 2003). Liposomes with two different compositions were prepared. The first composition was DPPC:MPPC:DPPG:DSPE-PEG(2000):DSPE-PEG(3400)-NHS at a molar ratio of 82:7:4:4:4 (calcein-liposomes). The second composition was DPPC:MPPC:RhoPE:DSPE-PEG(3400)- NHS: at a molar ratio of 86:7:3:4 (rhodamine-liposomes). All lipids were dissolved in chloroform. A thin lipid film was formed by drying the lipids under nitrogen at 46°C for 2 hours. Lipids were hydrated in 1 ml of distilled water at 46°C for 4 minutes and filtered through a 220nm filter system using a gas-tight sample-Luer Lock syringe from Hamilton (Reno, USA). A 0.5ml aliquot of trastuzumab in water at a concentration of 1.5 mg/ml was added to the filtered lipid. The trastuzumab amine binds to the DSPE-PEG(3400)-NHS ester to form an amide bond that links the antibody to the liposome (Kullberg et al. 2003, Wei et al. 2003). For control experiments, calcein-liposomes were also prepared without the addition of trastuzumab.

All liposomes were incubated at 46°C for 10 minutes and left at 4°C overnight to allow conjugation of the trastuzumab. Liposomes were then extruded 10 times at 46°C using an extruder from Eastern Scientific (New York, USA). The calcein-liposomes were extruded through a 100 nm filter and rhodamine-liposomes were extruded through a 200nm filter. All liposomes were put through three freeze-thaw cycles alternating between 46°C and 0°C every three minutes.

3.3.4 *Calcein-Liposomes*

A 0.15ml aliquot of liposomes was added to 0.15ml of a 320mM calcein solution at pH 6.1. The liposomes were heated to 41.5°C for 5 minutes. During this heating, the MPPC forms pores in the liposomes causing an influx of calcein. Filtering through a CL-4B Sepharose column at room temperature removes both unencapsulated calcein and unconjugated trastuzumab. Liposomes were stored at 4°C.

3.3.5 *Rhodamine-Liposomes*

A 0.15ml aliquot of liposomes was mixed with 0.15ml of a 1xPBS solution and heated to 41.5°C for 5 minutes. The liposomes were run through a CL-4B Sepharose column to remove unconjugated trastuzumab, and the solution was stored at 4°C.

3.3.6 *Liposome characterization*

Using a Stewart lipid concentration assay and a BCA protein concentration assay, it was determined that the protein to lipid ratios of trastuzumab to calcein-liposomes and trastuzumab to rhodamine-liposomes were 0.15 (w/w) and 0.17 (w/w), respectively (Krohn 2002, Torchilin and Weissig 2003). Liposomes were sent to Northern Lipids (Burnaby, Canada) for sizing. Vesicle size was determined using a Malvern Zetanano sizer ZS90 with Dispersion Technology Software version 5.03 following NLI standard operating procedure OE-109-00.

3.3.7 *Temperature induced release of calcein in vitro*

Three sets of calcein-liposomes were produced independently and tested to characterize the temperature-induced release of calcein. The liposomes at room temperature were heated to temperatures ranging from 33°C to 45°C for four minutes in either 1x PBS or 50% fetal bovine serum. Liposome incubation in 50% serum will expose the liposomes to serum proteins and should give a better indication of liposome behavior *in vivo*. A long term stability test was performed by incubating calcein-liposomes at 37°C in either PBS or 50% serum for time intervals up to 8 hours. After incubation, the sample volume was brought to 3ml with a 1x PBS solution containing 2mM EDTA, added to prevent calcein quenching by endogenous divalent cations. Fluorescence was measured using a Perkin Elmer LS50B luminescence spectrometer with an excitation wavelength of 490 nm, an emission detection wavelength of 515 nm and slit widths set at 2.5 nm. Percent leakage from the liposomes was determined using the formula:

$$\% \text{ calcein release} = (F - F_1)/(F_T - F_1) \times 100$$

F_1 is the initial fluorescent reading, F is the fluorescent reading after incubation at a given temperature in either PBS or 50% serum, and F_T is the fluorescent reading after complete release from the liposomes. Complete release was triggered by adding 15µl of 10% Triton X-100 to the 3 ml volume to cause liposome disruption.

3.3.8 *Intracellular calcein release*

Internalization studies were performed using ce2 cells grown on 6-well chamber slides (Nalge Nunc International, Rochester, USA) in 0.4 ml of DMEM medium plus additives. The medium was removed 24 to 48 hours after subculture and replaced with 0.4ml of medium containing 7.5 µg of calcein-liposomes and 3.5 µg of rhodamine-liposomes. Cells were incubated in the presence of the liposomes for 75 minutes at 37°C and 5% CO_2. After incubation, the liposome-containing medium was removed from the cells and replaced with fresh medium without liposomes. The chamber slide was then submerged

in a 42°C water bath for four minutes. A temperature of 42°C rather than the optimum release temperature of 40.5°C (Figure 3.2A), was used to help ensure that the internalized liposomes were heated through their lipid transition temperature in the given time. Four control experiments were run in parallel to this experiment to insure that the fluorescence was due to Her-2 specific internalization and temperature triggered release. The experimental study and controls are outlined in Table 3.1.

Table 3.1. Conditions for intracellular calcein release experiments.*

Description	**Media Additives**†	**Incubation Temperature**‡
Control 1: no liposomes	no additives	42°C
Control 2: liposomes without conjugated trastuzumab	calcein-liposomes without conjugated trastuzumab, rhodamine-liposomes	42°C
Control 3: excess free trastuzumab	calcein-liposomes, rhodamine-liposomes, 1mg/ml of unconjugated trastuzumab	42°C
Control 4: 37°C	calcein-liposomes, rhodamine-liposomes	37°C
Experimental	calcein-liposomes, rhodamine-liposomes	42°C

* Ce2 cells were incubated in 0.4 ml of medium with the specified additives for 75 minutes at 37°C.

† When included as an additive, calcein-liposomes and rhodamine liposomes were added at weights of 7.5 μg and 3.5 μg respectively. All calcein-liposomes and rhodamine-liposomes were conjugated to trastuzumab unless otherwise noted.

‡ Cells were heated to either 37°C or 42°C for four minutes after a 75-minute incubation with the additives.

After heating, the cells were rinsed once with 1x PBS and imaged in a 2.5% glycerol-PBS solution using a Leica DMI6000 B inverted fluorescence microscope. To measure the internalization and release of calcein-liposomes, the amount of fluorescence from the calcein was quantified. Quantification was performed on three slides with three images taken on each slide. The nine image fields were chosen based on cell health and density before imaging with fluorescence. Cells were not fixed in order to show intracellular calcein release within live cells. For each of the conditions in Table 3.1, over 100 cells were analyzed to determine the average cellular calcein signal. Although data analysis was performed only on calcein fluorescence, cells were incubated with both calcein-liposomes and rhodamine-liposomes to mimic the conditions of the two-component experiments.

3.3.9 *Analysis of two-component overlap*

The method for the two-component overlap experiment was similar to the experimental conditions outlined in the final row of Table 3.1. The only difference was that after the 75 minute incubation, the cells were fixed in 4% paraformaldahyde for 15 minutes. The advantage of fixing the cells is that they remain spread out on the plate, allowing for a more accurate analysis of fluorescence overlap. The fixation also reduces cell depth, enabling more efficient imaging in the z-plane and brighter overall fluorescence. Following fixation, the cells were submerged in a 42°C water bath for four minutes. Cells were rinsed once with 1x PBS and visualized in a 2.5% glycerol-PBS solution, using a Leica DMI6000 B fluorescent microscope.

3.3.10 *Fluorescence intensity measurements*

Images were taken with a Leica DMI6000 B inverted fluorescent microscope using a 40x objective and 10x ocular. For each image field, 7 photographs were taken at different depths along the z-axis, with distance between the z-planes being 2-3 μm. The 7 photographs were deconvolved with Leica deblur software. Using an adaptive threshold

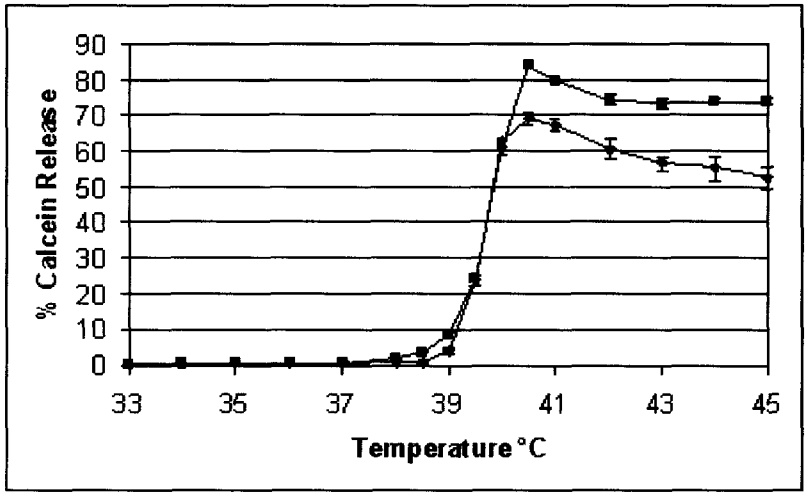
option in the software, background and halo fluorescence were subtracted from the image. The only remaining fluorescence was the punctuate signal from calcein or rhodamine encapsulated in endosomes. To measure the fluorescence of a cell, we used NIH-Image J software with modifications in the open source Java code. Each fluorescent object in the cell of interest was located with the software and analyzed independently to determine its total fluorescence. For the overlap experiments, the program analyzed each object of green calcein fluorescence and then determined if it was overlapped with an object of red rhodamine fluorescence. In order to be classified as an overlap, the objects had to be superimposed in the x-y plane and have their maximum intensity in the same z-plane. Both the ce2 and MTSV1-7 cells displayed a subtle amount of auto-fluorescence. To eliminate objects that were simply auto-fluorescence artifacts, we utilized the blue fluorescence measurement. Neither the rhodamine nor calcein probes gives any signal in blue fluorescence but an auto-fluorescent object fluoresces in red, green and blue. By having the program skip any object with blue fluorescence, we were able to restrict artifacts due to auto-fluorescence.

3.4 Results

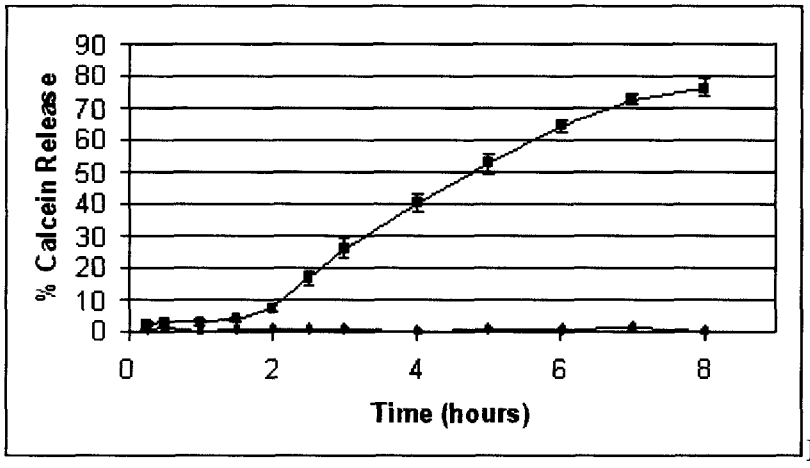
3.4.1 *Characterization of thermosensitive liposomes*

The average diameters of the calcein-liposomes and rhodamine-liposomes were measured as 124 nm and 167 nm, respectively. Both sets of liposomes were found to have narrow size distribution, showing polydispersity indices of less than 0.1. To determine their temperature release profile, the calcein-liposomes were incubated for four minutes at temperatures ranging from 33°C to 45°C in either 1x PBS or 50% serum. As shown in Figure 3.2, the calcein-liposomes release less than 1% of their contents at 37°C. Peak release is at 40.5°C with calcein-liposomes releasing 69% of the calcein in PBS and 84% in 50% serum.

The stability of liposomes at body temperature was tested by incubating liposomes for up to 8 hours at 37°C (Figure 3.2B). In 1x PBS, liposomes remain stable for over 8 hours, releasing only 1% of their contents. However, when incubated in 50% serum, the liposomes become steadily leakier over time. At 2 hours the liposomes have leaked only 7% of their contents, but by 8 hours over 75% of the entrapped calcein has escaped into the surrounding buffer.



A



B

Figure 3.2. Temperature triggered release of calcein from calcein-liposomes. Composition of the calcein-liposomes is given in Materials and Methods. Data (mean ± SE) represent fluorescence spectrometric measurements from triplicate sets of calcein-liposomes. A, Percentage of total calcein released after heating for four minutes in either

PBS ♦ or 50% serum ■. B, Percentage of total calcein that leaked at 37°C in either PBS ♦ or 50% serum ■ after extended incubation.

3.4.2 *Intracellular heat-triggered calcein release from liposomes*

Since the calcein-liposomes are conjugated to trastuzumab, they are predicted to bind specifically to the Her-2 receptors located in the plasma membranes of target cells. After binding, the liposome-receptor complex will be directed to clathrin-coated pits and internalized into cellular endosomes. Each calcein-liposome contains a 160 mM calcein solution. Because calcein is self quenching at this high concentration, the internalized calcein-liposomes will give only a limited signal when visualized with fluorescence microscopy. However, when the calcein-liposomes are subsequently heated to 42°C, the calcein releases from the liposome and should fill the endosomal compartment. Dilution of the calcein restores its fluorescence, allowing for quantification of the temperature-triggered release.

To study the cellular targeting and internalization of calcein-liposomes, we utilized Her-2 expressing mammary epithelial ce2 cells. The ce2 cells were incubated with calcein-liposomes for 75 minutes at 37°C and then heated to either 37°C or 42°C for 4 minutes. Visualization with fluorescence microscopy shows the effect of temperature on intracellular release of calcein from calcein-liposomes in ce2 cells. While there is some signal apparent in the cells at 37°C, there is a marked increase in calcein fluorescence after heating the cells to 42°C (Figure 3.3). Analysis with NIH Image J software of over 100 cells for each experimental condition shows that this increase is approximately 7-fold (Figure 3.4).

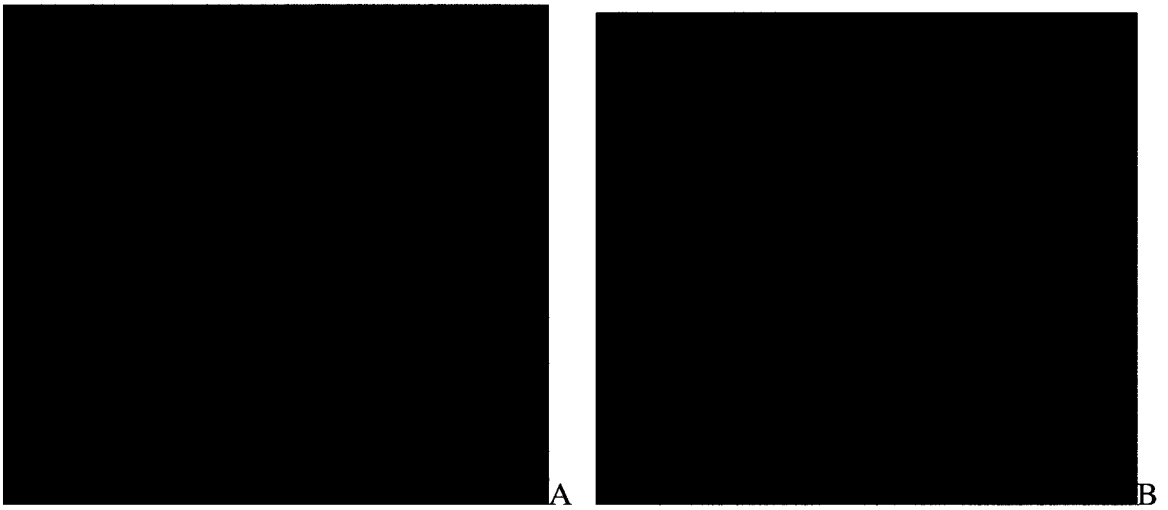


Figure 3.3. Temperature induced release of calcein from calcein-liposomes in ce2 cells. Ce2 cells were incubated with calcein-liposomes and rhodamine-liposomes and heated to either 37°C (A) or 42°C (B) . Green points represent calcein fluorescence (rhodamine fluorescence not shown). A punctuate calcein signal was assumed to emanate from an endosome, because free calcein released into the cytoplasm from endosomes would be undetectable. Heating to 42°C releases high concentration calcein from liposomes into the endosomal compartments, causing a dilution of the self-quenching calcein and therefore an increase in fluorescence. The calcein signal is 7-fold greater (Figure 3.4) upon heating to 42°C versus 37°C. Images show unfixed cells and were taken at a magnification of 400x.

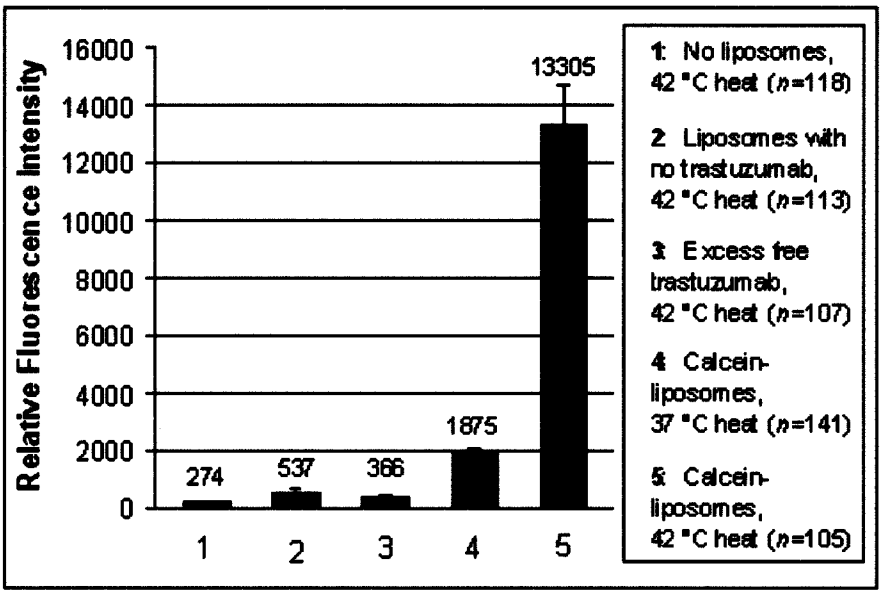


Figure 3.4. Intracellular heat release of calcein-liposomes. Data (mean ± SE) were obtained from measurement of calcein fluorescence in ce2 cells after incubation under conditions described in Table 3.1. The number of cells analyzed (n) for each experimental condition is given in the figure box. The number above each data bar shows the relative fluorescence intensity of calcein signal. Bars 4 and 5 show that after incubation with calcein-liposomes, cells that have been heated to 42°C have a 7-fold increase in relative fluorescence compared to cells that been heated to 37°C

Baseline autofluorescence of ce2 cells was determined by incubating ce2 cells without any liposomes, followed by heating to 42°C for four minutes and analysis with fluorescence microscopy. Results show that the ce2 cells have an average background fluorescence of 274 (Figure 3.4). In addition, two control experiments were run to determine if the liposome internalization was Her-2 specific. First, calcein-liposomes were prepared without trastuzumab conjugated to their surface. After a 75-minute exposure to these liposomes and heating to 42°C, the ce2 cells display an average fluorescence of 537. This is a 24-fold reduction from the fluorescent signal of cells that were exposed to trastuzumab-conjugated calcein-liposomes (Figure 3.4). The second control for Her-2 specificity was a competitive binding assay. Cells were incubated with

the trastuzumab-conjugated calcein-liposomes and an excess of free trastuzumab. The concept of this competitive assay is that free trastuzumab should overwhelm the Her-2 binding sites and restrict the sites available for binding of calcein-liposomes conjugated to trastuzumab. If the internalization of calcein-liposomes is Her-2 specific, then restriction of binding sites should limit liposome endocytosis. Indeed, the addition of free trastuzumab lowers the calcein signal by over 36-fold (Figure 3.4).

3.4.3 *Two-component overlap*

The effectiveness of a two-component delivery system was tested using ce2 cells and control MTSV1-7 cells. These cell lines are identical except that the ce2 cells have been transfected with Her-2 DNA and therefore overexpress the receptor by 10-fold. For a two-component therapy to be effective, it must increase the specificity of drug delivery to ce2 cells relative to MTSV1-7 cells. In the present study, trastuzumab-conjugated calcein- and rhodamine-liposomes represented a two-component delivery system. Effective drug delivery was defined as co-localization of calcein- and rhodamine-liposomes within the same endosome. An endosome was assumed to contain at least one rhodamine-liposome and one calcein-liposome if a calcein and rhodamine signal overlapped in the x, y and z planes after heating to 42°C for four minutes. Visualization of the two cell lines after incubation with the two types of liposomes reveals endosomes containing green calcein signals and red rhodamine signals (Figure 3.5). A yellow signal indicates an overlap of at least one calcein-liposome and one rhodamine-liposome in a single endosome.

Figure 3.5. Two-component overlap in ce2 cells versus MTSV1-7 cells. Ce2 cells (A) and MTSV1-7 cells (B) have internalized calcein-liposomes (green) and rhodamine-liposomes (red). Cells are shown after incubation at 37°C for 75 minutes, followed by heating to 42°C for 4 minutes in order to release calcein from calcein-liposomes. Overlap of a rhodamine-liposome and calcein-liposome results in a yellow point. Analysis shows that an overlap of calcein-liposomes and rhodamine-liposomes is much more frequent in ce2 cells (Table 3.2). Images show fixed cells and were taken at a magnification of 400x.

Calcein fluorescence was used to represent the quantity of drug being delivered to a cell. As an index of the amount of drug that would be delivered to a cell by a one-component therapy, we quantified the total fluorescence of calcein within the cell. To measure the amount of drug that would be delivered with a two-component approach, we needed to quantify the amount of calcein from endosomes that contained both a calcein signal and a rhodamine signal. These endosomes were detected using NIH Image J software and their total calcein signal within a cell was used as an index of the drug that would be delivered with a two-component therapy. The results, shown in Table 3.2, indicate that a two-

component therapy would deliver approximately 35-fold more drug to Her-2 overexpressing ce2 cells than to control MTSV1-7 cells, whereas a one-component targeted therapy would deliver approximately 5-fold more drug to ce2 cells than to MTSV1-7 cells. The ratio of these numbers indicates a 7-fold increase in specificity achieved by a two-component system compared to a one-component system.

Table 3.2. Increased specificity for ce2 cells using a two-component therapy. *

	Calcein †	**Calcein Overlapped with Rhodamine**‡	**Percentage of Calcein Overlapped with Rhodamine** ¶
CE2 Cells (*n*=83)	21264 ± 2261	7885 ± 956	31.2% ± 1.7%
MTSV1-7 cells (*n*=70)	4307 ± 611	221 ± 64	3.5% ± .9%
Ratio of CE2/MTSV1-7 §	4.9	35.6	8.8

* Data (mean ± SE) represent relative fluorescence intensity of calcein per cell. The number of cells analyzed (*n*) is indicated for each cell type.

† Data represent the amount of drug that would be delivered with a one-component targeted drug delivery system.

‡ Data represent the amount of drug that would be delivered using a two-component targeted drug delivery system.

¶ Data show the percentage of calcein signal that is overlapped with rhodamine signal. Numbers in the first and second columns give an indication of total fluorescence summed over all the cells and then averaged; whereas in the third column, the percentage overlap is calculated individually for each cell before averaging so that every cell is given the same influence on the mean.

§ The bottom row shows the ratio of data from row 1 versus row 2, indicating the

specificity of delivery to Her-2 overexpressing ce2 cells relative to MTSV1-7 cells.

Another approach to analyzing the data is to calculate the percentage of calcein signal that is overlapped with rhodamine in each individual cell. This overlap percentage indicates what percentage of the total calcein signal would be delivered to the cell using a two-component technique. When the mean overlap percentage is calculated for a cell population, this method for analysis gives each cell an equal weight, whether the cell is quiescent or actively internalizing liposome-receptor complexes. The mean overlap percentages in MTSV1-7 cells and ce2 cells were found to be 3.5% and 31.2%, respectively (Table 3.2). The ratio of these percentages shows that a two-component therapy is 8.8-fold more effective than a one-component therapy in targeting Her-2 overexpressing ce2 cells.

3.5 Discussion

Chemotherapeutic drugs can only effectively treat a cancer if they are able to distinguish between normal cells and tumor cells. While there is an abundance of drugs that are cytotoxic, their lack of specificity for tumor cells reduces the tolerable dosage of treatments and makes it difficult to completely eliminate the cancer. This problem is a major shortcoming in current chemotherapeutic techniques. In this study we evaluate a drug delivery strategy that significantly increases targeting of Her-2 overexpressing cells and should therefore enhance the specific delivery of chemotherapeutic agents to tumor cells in the body.

Our first goal was to create a thermosensitive liposome conjugated to the antibody trastuzumab. Secondly, through the use of Her-2 overexpressing ce2 cells, we hoped to observe internalization and heat triggered release from the thermosensitive liposome into cellular endosomes. Finally, using these liposomes we wanted to test the merit of a two-

component therapy that would allow for more efficient targeting of Her-2 overexpressing cells. Our results show that it is possible to conjugate antibodies onto the surface of a thermosensitive liposome while retaining the heat release characteristics of the liposome (Figure 3.2). Calcein-liposomes are remarkably stable in PBS at 37°C. They show minimal calcein leakage after extended incubation at 37°C, but rapid release of their contents upon heating to 42°C. The liposome integrity becomes compromised, however, when incubated in the presence of serum at 37°C. The eventual leakage of thermosensitive liposomes in serum has been attributed to proteins penetrating the bilayer and reducing the liposome integrity (Scherphof et al. 1978, Hosokawa et al. 2003, Han et al. 2006, Hossann et al. 2007). Increasing the amount of either PEG or DPPG in the lipid bilayer has been shown to reduce leakiness by changing the interaction between these proteins and the liposomes (Hosokawa et al. 2003, Han et al. 2006). PEG sterically hinders the integration of proteins while DPPG interferes electrostatically. Since chemotherapeutic liposomes will contain toxic materials, the eventual leakage of thermosensitive liposomes in serum is an issue that needs further study.

Our results indicate that calcein-liposomes bind and internalize into ce2 cells through Her-2 specific endocytosis (Figure 3.4). Furthermore, after internalization, the liposomes can be induced to release their contents into endosomal compartments after heating to 42°C. Since tumor hyperthermia is a well-developed field, many external and interstitial applicators are available that use electromagnetic energy to heat specific tissues (Diederich 2005, Haemmerich and Laeseke 2005, Stauffer 2005, Demura et al. 2006, Hauck et al. 2006). With this technology, it should be possible to restrict release from thermosensitive liposomes specifically to the tumor region.

All chemotherapies must contend with the irregular vasculature of solid tumors, which impedes drug delivery to cancer cells. In this respect, a potential benefit of hyperthermia is to enhance the permeability of tumor vessels. Raising tumor temperature to 40-42°C for 1 hour results in a subsequent 5 hour period where extravasation of 100 nm liposomes

is dramatically increased (Kong et al. 2000, Kong et al. 2001). However, even with hyperthermia-enhanced penetration, high liposome concentrations may be required to achieve drug delivery deep within the tumor. A highly specific targeting system, such as the one proposed here, should permit an elevated dosage of liposomes and a more effective treatment of the entire tumor.

The theory behind a two-component therapy is that drug will only be delivered to the cytoplasm of a target cell if two liposomes interact in the same endosomal compartment. Provoda et al. created a one-component delivery system by co-encapsulating the pore forming protein LLO and the toxin gelonin into a single set of pH-sensitive liposomes (Provoda et al. 2003). It should be possible to create a similar system, but with LLO and gelonin encapsulated into separate sets of thermosensitive liposomes targeted to Her-2. Only if the two sets of liposomes colocalize in an endosome and are exposed to an external heat source will gelonin reach the cell cytoplasm where it can kill the cell (Figure 3.1). Using rhodamine-liposomes and calcein-liposomes, we tested the hypothesis that a two component therapy increases specificity for Her-2 overexpressing cells. Our data indicate that a two-component therapy is 7- to 9-fold more effective than a one-component therapy in targeting cells that overexpress Her-2. Furthermore, the two-component system delivers 35-fold more calcein to Her-2 overexpressing ce2 cells than to normal MTSV1-7 cells (Table 3.2).

One drawback of using thermosensitive liposomes and localized hyperthermia is the limited ability to treat metastatic tumors that may have already disseminated to different regions of the body (Andresen et al. 2005, Ponce et al. 2006). Although the two-component therapy presented in this study has been developed using thermosensitive liposomes, functionality of the delivery system is not necessarily dependent on heat triggered release. By using trastuzumab conjugated pH-sensitive liposomes that rupture in the acidic endosomal compartments, one could create a systemic two-component therapy that would target Her-2 overexpressing cells throughout the body. In addition,

while our research has focused on the development of a two-component system, a three- or more component system operating on the same principles might further amplify the specificity of drug delivery to cancer cells overexpressing Her-2.

Chemotherapies are notoriously hard on cancer patients and their cytotoxic effects limit the doses that can be safely administered to a patient. The fluorescence-overlap experiments in this study show that a two-component drug delivery system would greatly increase specific targeting of Her-2 overexpressing cancer cells. By combining the tissue specificity of local hyperthermia with the cellular specificity of Her-2 targeting, these liposomes have the potential to be an effective drug delivery tool.

3.6 Acknowledgments

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3.7 Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Chapter 4: Listeriolysin O Enhances Cytoplasmic Delivery by Her-2 Targeting Liposomes[1]

4.1 Abstract

To enhance cytoplasmic delivery of liposomal contents to breast cancer cells, we have attached the pore-forming protein, listeriolysin O (LLO) to thermosensitive liposomes. The antibody trastuzumab (Herceptin®) was also conjugated to the outer surface of the liposomes, resulting in highly specific binding and internalization into mammary epithelial cells that overexpress the human epidermal growth factor receptor 2 (Her-2). The liposomes were preloaded with a marker fluorescent dye, and the effect of LLO on the distribution of dye within the cells was monitored using fluorescence microscopy. Due to the thermosensitive nature of the liposomes, hyperthermia at 42°C triggered release of the encapsulated fluorescent calcein from the endocytosed liposomes into the interior of the endosomes. LLO, when conjugated to these liposomes, subsequently formed pores in the endosomal membrane, allowing calcein to flow out of the endosomal compartment into the cytoplasm. Her-2 targeted liposomes bearing LLO delivered a 22-fold greater concentration of calcein to mammary epithelial cells that overexpress Her-2 compared to cells with normal Her-2 expression. Thus, the addition of LLO to preformed liposomes offers a method for significantly enhancing delivery of liposomal contents to the cytoplasm of targeted cells.

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4.2 Introduction

Penetration of the endosomal barrier and delivery of macromolecules to the cell cytoplasm remains an obstacle for liposomal delivery systems (Belting et al. 2005, El-Sayed et al. 2009). Liposomes that have been endocytosed into target cells are quickly routed to lysosomes where, without a mechanism for release into the cytoplasm, their encapsulated cargo is broken down by lysosomal degradative enzymes. Given the importance of compromising the endosomal membrane for drug delivery, several techniques have been developed to address the challenge.

Methods for increasing cytoplasmic delivery include the conjugation of viral components or pH sensitive lipids to liposomes, thereby enhancing the likelihood of the liposome fusing with the endosomal membrane upon endosome acidification (Karanth and Murthy 2007). Another approach involves the addition of membrane-disruptive polymers and light- activated photosensitizers which cause endosomal breakdown and increase cytoplasmic delivery from liposomes (Fretz et al. 2007, El-Sayed et al. 2009). In this paper we modify a technique described by Lee et al. (Lee et al. 1996, Mandal et al. 2004) who encapsulated the pore-forming protein, Listeriolysin (LLO), within pH-sensitive liposomes and demonstrated enhanced delivery of macromolecules from liposomes to the cellular cytoplasm. LLO is a 58 kDa protein that utilizes cholesterol to form pores in the endosomal membrane, enabling escape of the endocytosed bacterium *Listeria monocytogenes* into the cytoplasm of infected cells (Schnupf and Portnoy 2007).

To simplify the addition of LLO to liposomes we have taken advantage of LLO's ability to bind stably to the lipid bilayer of liposomes (Jacobs et al. 1998, Bavdek et al. 2007). Instead of encapsulating LLO in the lumen of the liposomes, as done by Lee et al. (1996), we added LLO to preformed cargo-laden liposomes and allowed it to conjugate directly to the lipid bilayer. When the liposomes are internalized into target cells, the

attached LLO is also endocytosed. The cholesterol-containing endosomal membrane and low pH environment allow the LLO to form pores in the endosome and thereby facilitate delivery of the liposome cargo to the cytoplasm.

In a previous study (Kullberg et al. 2009), we targeted Her-2 overexpressing mammary epithelial cells using thermosensitive liposomes (Needham et al. 2000) conjugated to the antibody Trastuzumab. The liposomes bound and internalized into Her-2 overexpressing cells with high specificity, and released an encapsulated fluorescent marker into the cellular endosomes in response to hyperthermia. However, the fluorescent marker was predominantly trapped in the endosomes. To achieve passage of agents from the endosome to the cytoplasm, a further modification of the delivery system was necessary. In this study, the ability of the LLO-liposomes to deliver their contents to the cytoplasm of Her-2 overexpressing mammary epithelial cells was tested *in vitro*.

4.3 Materials and Methods

4.3.1 *Materials*

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (MPPC) and 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DPPG) were purchased from Avanti Polar Lipids (Alabaster, USA). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[poly(ethylene glycol)-3400]-N-hydroxy succinamide (DSPE-PEG(3400)-NHS) was purchased from Shearwater Polymers (Huntsville, USA), now Nektar Therapeutics (San Carlos, USA). CL-4B Sepharose gel, used for purification of the liposomes, was purchased from Amersham Biosciences (Uppsala, Sweden). Biotinylated His-Tagit Western blotting system was purchased from Calbiochem (San Diego, USA). ImmunoSelect streptavidin-alkaline phosphatase kit and Dulbecco's phosphate-buffered saline (DPBS) were bought from Gibco BRL (Gaithersburg, USA). The cell lines MTSV1-7 and ce2 were kindly supplied by Dr. Joyce Taylor-Papadimitriou at the Breast Cancer Biology Group, King's College London School of Medicine, U.K. Dr. Max Rabinowitz at Alaska Oncology and Hematology,

Anchorage, AK generously donated trastuzumab. LLO-pEt29-DP-E3570 transfected E. coli were provided by Dr. Dan Portnoy at University of California, Berkeley, CA. All other chemicals and reagents were purchased from Sigma Chemicals Company (USA).

4.3.2 *Cell culture*

The human mammary epithelial cell lines MTSV1-7 and ce2 were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum and supplements as previously described (Kullberg et al. 2009).

4.3.3 *Protein purification*

LLO was purified following a previously described protocol (Glomski et al. 2002) with slight modifications to the technique and buffers. Instead of using a French press, bacteria were probe sonicated to disrupt the bacterial membranes. The buffers used in this purification were sonication buffer [50mM phosphate buffer, pH 8.0, 1M NaCl, 10mM ß-mercaptoethanol, 1mM phenylmethylsulfonyl fluoride (PMSF)], wash buffer [50mM phosphate buffer, pH 6.0, 1M NaCl, 10 mM ß-mercaptoethanol, 0.1% Tween-20, 10% glycerol], elution buffer [50mM phosphate buffer, pH 6.0, 1M NaCl, 10mM ß-mercaptoethanol, 800mM imidazole] and storage buffer [50mM phosphate buffer, pH 6.0, 1M NaCl, 1mM EDTA 5mM dithiothreitol].

4.3.4 *Preparation of liposomes*

Liposomes were prepared by the film hydration-extrusion method (Torchilin and Weissig 2003). Liposomes were made with lipids DPPC:MPPC:DPPG:DSPE-PEG(3400)-NHS at a molar ratio of 82:10:3.5:4. The procedure for conjugating liposomes to trastuzumab and filling liposomes with calcein was carried out as previously described with the exception that in this study liposomes were filtered at 200 nm instead of 100 nm (Kullberg et al. 2009). Liposomes with encapsulated calcein were run over a CL-4B column equilibrated with DPBS pH 7.4 to remove unencapsulated calcein and any

unconjugated trastuzumab. The peak fractions were identified on the basis of their calcein content and pooled.

4.3.5 *Conjugation of LLO to liposomes*

An 8µl aliquot of LLO (2.0 mg/ml storage buffer) was added to 0.4 ml of CL-4B purified liposomes at a lipid concentration of 0.33 mg/ml. The mixture was left at room temperature for 10 min and then 8 µl of cholesterol (25 mg/ml in 200-proof ethanol) was added to the solution. The liposomes were rotated slowly for 30 min at room temperature and then centrifuged at 3800 g for 3 min to pellet the cholesterol. The supernatant containing liposomes was run over a CL-4B column one more time to remove any unconjugated LLO or cholesterol that remained. The peak fractions were pooled, and liposomes (LLO-liposomes) were normalized to a lipid concentration of 0.18 mg/ml and stored at 4°C until used. The liposomes were centrifuged again at 3800 g for 3 min prior to cell culture experiments to ensure no aggregates remained. Control liposomes (control-liposomes) without LLO were made following an identical procedure except that 8 µl of LLO storage buffer was added instead of 8 µl of LLO.

4.3.6 *Liposome sizing*

Liposomes were sent to Northern Lipids (Burnaby, Canada) for sizing. Vesicle size was determined in triplicate samples using a Malvern Zetanano sizer ZS90 with Dispersion Technology Software version 5.03 following NLI standard operating procedure OE-109-00.

4.3.7 *Column chromatography and LLO Western detection*

As liposomes were run through the final CL-4B column, fractions of 0.25 ml each were collected. To determine the liposome peak, the first 11 fractions were tested for entrapped calcein. A 10 µl aliquot of the fraction was put in 3 ml DPBS and liposomes were ruptured by adding 10 µl of 10% Triton X-100 to the 3 ml volume. Calcein was detected as previously described (Kullberg et al. 2009). To determine LLO content, the

fractions were electrophoresed on a 7.5% SDS-polyacrylamide gel and blotted onto PVDF membrane. LLO was detected using biotinylated His-tag primary antibody, a streptavidin alkaline phosphatase secondary antibody, and NBT/BCIP staining of the blot.

4.3.8 *Liposome pull down*

Liposomes were conjugated to protein A Sepharose beads through binding of protein A to trastuzumab on the liposomes. 10 mg of beads were hydrated in 50 µl of DPBS and 50 µl of liposome solution was added to the hydrated beads. The solution was rotated slowly for 3 hours at room temperature and then centrifuged at 240 g for 3 min to pellet the beads. The supernatant was removed and saved for analysis, and the beads were rinsed in 1.3 ml of DPBS. The rinse was repeated 5 times, and the beads were resuspended in DPBS at their initial volume. The samples were diluted in 2x SDS-PAGE loading buffer and LLO was eluted off the beads by boiling for 3 minutes. LLO was detected using Western immunoblot analysis as already described for the column fractions.

4.3.9 *Temperature induced release of calcein*

After filtering liposomes at 200 nm, three sets of LLO-liposomes and three sets of control-liposomes were made. The liposomes were heated to temperatures ranging from 35°C to 45°C for two minutes in 1x DPBS. Percent leakage from the liposomes was determined using previously described methods (Kullberg et al. 2009).

4.3.10 *Cytoplasmic fluorescence measurement*

Cytoplasmic delivery of calcein to ce2 and MTSV1-7 cells was observed directly using fluorescence microscopy. The ce2 and MTSV1-7 cells were grown on 8-well chamber slides (Nalge Nunc International, Rochester, USA) in 0.4 ml of DMEM medium/well. The medium was removed 24 to 48 hours after subculture and replaced with a solution comprising 0.1 ml of complete medium and 0.1 ml of either LLO- or control-liposomes

in DPBS at a lipid concentration of 0.18 mg/ml, such that the final lipid concentration was 0.09 mg/ml. Cells were incubated in the presence of the liposomes for 1.5 hrs at 37°C and 5% CO_2 before liposomes were removed and replaced with liposome-free medium. For the 1.5 hour time point, the slide chamber was then submerged in a water bath at either at either 37°C or 42°C for four minutes. For the 4 hour and 10 hour time points, the cells were incubated an additional 2.5 or 8.5 hours at 37°C and 5% CO_2, followed by submersion in a 37°C water bath for four minutes. Following submersion in the water bath, cells for all time points were then incubated at 37°C and 5% CO_2 for an additional 10 minutes, rinsed once with 1x DPBS, fixed with 4% para-formaldehyde at room temperature and imaged using a Leica DMI6000 B inverted fluorescence microscope. Each experimental condition was replicated on three separate slides and three image fields were recorded from the respective well on each slide, resulting in nine image fields for each treatment for each cell line. The image fields were chosen based on cell health and density before imaging with fluorescence.

4.3.11 *Quantification of cytoplasmic fluorescence*

The nine image fields corresponding to each experimental condition were analyzed using a combination of options from Leica Deblur software and NIH-Image J software. Before observing the fluorescent image, the center of each cell in a brightfield image was overlaid with a circle of area 400 μm^2 in which the cytoplasmic fluorescence would be measured. To accurately measure the diffuse cytoplasmic fluorescence, it was first necessary to remove any intense puncate signal emanating from calcein-filled endosomes within this circle. Using an adaptive threshold option in the Leica Deblur Software, we were able to identify the puncate signals and remove them from the image field. The remaining pixels within the circle were analyzed with NIH-Image J software to determine the average fluorescence of the cytoplasm in this region. Background fluorescence was measured from three separate cell-free regions of the image field, averaged, and subtracted from the cytoplasmic fluorescence measurement. The fluorescence measurements were averaged from all cells within the respective 9 image fields, giving a

final fluorescence reading that represents the average concentration of calcein delivered to the cytoplasm in each of the experimental conditions and in each of the cell lines.

4.4 Results

4.4.1 *Characterization of LLO-liposomes*

Western blot analysis of purified LLO protein showed a clean product with a molecular weight of 59.5 kDa, in close proximity to the predicted 58 kDa (Schnupf and Portnoy 2007). Addition of this LLO product to the liposome surface increased the diameter of the liposomes from 213 ± 8 nm to 235 ± 5 nm. After LLO was attached to the liposomes, any unbound LLO was inactivated by incubation with cholesterol, a technique which has been shown to remove the pore forming ability of LLO (Jacobs et al. 1998, Gekara et al. 2005). The LLO-liposomes were then purified using column chromatography. Western immunoblot analysis of the column fractions showed that the concentration of LLO in each fraction corresponded closely to the concentration of liposomes, providing evidence that the LLO and liposomes were attached (Figure 4.1).

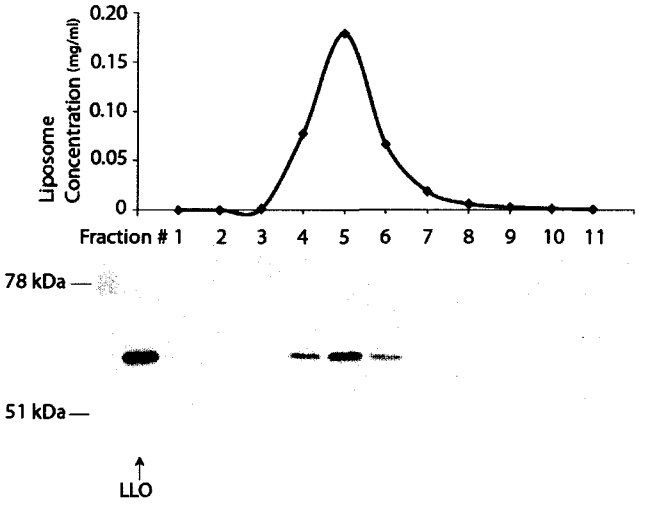

Figure 4.1. Western immunoblot analysis of LLO and liposomes in column fractions. CL-4B column fractions were collected and each fraction was analyzed for liposome concentration and for the presence of LLO. The liposome concentration is shown in graphical form (upper panel), and the corresponding Western blot analysis shows LLO

detected by His-tag antibody staining (lower panel). The first lane of the blot contains size markers, the second lane contains a sample of purified LLO and the remaining lanes contain column fractions 1-11. Peak concentrations of both liposomes and LLO were detected in fraction 5. A pull down assay was performed on liposomes from fraction 5 to determine if LLO and liposomes were conjugated (Figure 4.2).

To verify that the LLO was bound to liposomes, a pull-down assay was performed, conjugating liposomes to sepharose beads through trastuzumab-protein-A interactions. We reasoned that if LLO were not bound to the liposomes, the LLO would remain in the supernatant when the liposomes were removed with the beads. However, The LLO pulled down almost completely with the liposome conjugated beads, confirming that LLO and the liposomes are attached (Figure 4.2).

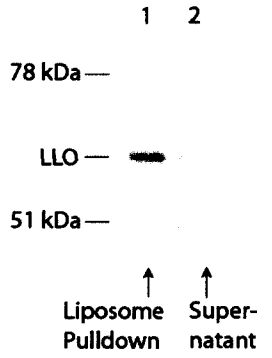

Figure 4.2. Pull down assay to verify LLO-liposome conjugation. Liposomes from the peak fraction (shown in Figure 4.1) were bound to protein A sepharose beads through trastuzumab-protein A interaction. After extensive rinsing, the settled beads and initial supernatant were analyzed for LLO content on a 7.5% SDS-polyacrylamide gel, followed by Western blot analysis on PVDF membrane. The vast majority of LLO is found with the liposome bound beads, verifying that LLO is attached to the liposomes.

Since the LLO is directly interacting with the liposome membrane, it could possibly be stabilizing or destabilizing the lipid bilayer, changing its thermosensitive properties relative to those of calcein-containing liposomes without LLO (control-liposomes).

Release of calcein from the liposomes was therefore tested at temperatures ranging from 35°C to 44°C. Results showed that the control-liposomes and LLO-liposomes had a similar thermosensitive profile (Figure 4.3). Both sets of liposomes were stable at body temperature, 37°C, but quickly released their contents when heated above 39.5°C.

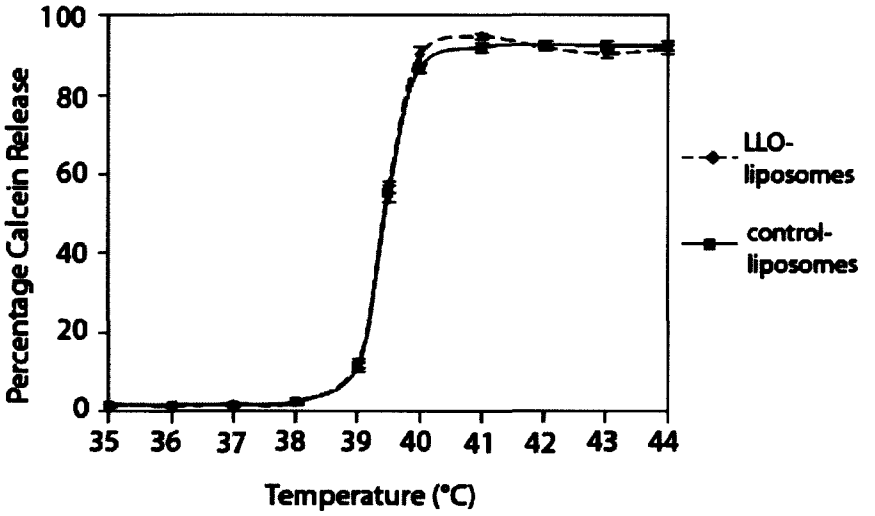

Figure 4.3. Temperature triggered release of calcein from control-liposomes and LLO-liposomes. The percentage of total calcein released from the liposomes was determined after 2 minutes heating at a given temperature. Data (mean ± SE) represent fluorescence spectrometric measurements from triplicate sets of LLO-liposomes (diamonds) and control-liposomes (squares).

4.4.2 *Heat-triggered cytoplasmic delivery from LLO-liposomes*

The ability of trastuzumab-coupled LLO-liposomes to deliver calcein to the cytoplasm was tested using two mammary epithelial cell lines, ce2 and MTSV1-7. These cell lines are identical except that ce2 has been permanently transfected with Her-2 DNA and expresses 10-fold more receptor than MTSV1-7 (D'Souza et al. 1993, Worthylake et al. 1999). Cells were exposed to the LLO-liposomes for 1.5 hours and then heated to either 37°C or an elevated temperature, 42°C, to trigger release of calcein from the liposomes. In addition, both sets of cells were exposed to control-liposomes and heated to 37°C and 42°C.

For each experimental condition, fluorescence microscopy analysis was performed on 9 image fields with a total of over 148 cells to determine the relative cytoplasmic concentrations of calcein (Table 4.1 and Figure 4.4).

Table 4.1. Hyperthermia-triggered cytoplasmic delivery of calcein*

	MTSV1-7	**Ce2**	**Ratio of Ce2 to MTSV1-7†**
37°C control-liposomes	2.03 ± 0.14 (n = 169)	4.21 ± 0.36 (n = 160)	2.07
42°C control-liposomes	1.56 ± 0.14 (n = 167)	13.98 ± 0.96 (n =194)	8.96
37°C LLO-liposomes	7.62 ± 0.44 (n = 148)	15.49 ± 0.65 (n = 172)	2.03
42°C LLO-liposomes	4.16 ± 0.35 (n = 159)	93.42 ± 4.14 (n = 160)	22.45

*Data (mean ± SE) represent relative fluorescence intensity of cytoplasmic calcein per cell after 1.5 hour exposure to liposomes. The number of cells analyzed (n) is indicated for each cell type and each experimental condition.

†This column shows the ratio of relative fluorescence intensities in ce2 versus MTSV1-7 cells for the given experimental conditions.

Control-liposomes showed very little cytoplasmic delivery to the cells at 37°C with a slightly elevated delivery to the Her-2 overexpressing ce2 cells when they were heated to 42°C. Adding LLO to the liposomes caused a small increase in cytoplasmic delivery to both cell lines at 37°C (Figure 4.4). At 42°C, the addition of LLO to the liposomes had a more significant effect, enhancing the cytoplasmic delivery to ce2 cells by 6.5 fold (Figures 4.4 and 4.5).

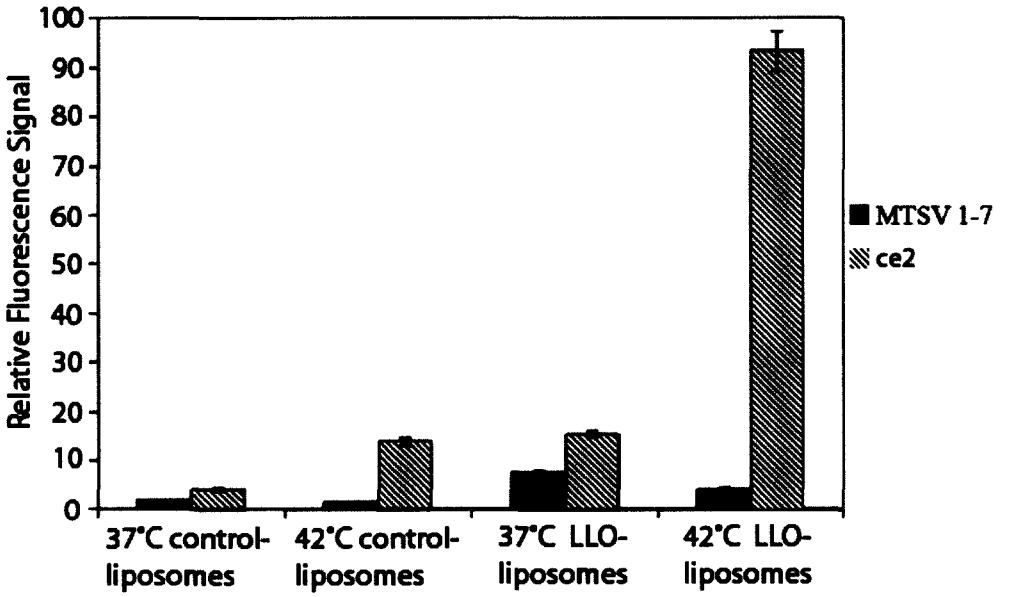

Figure 4.4. Cytoplasmic calcein delivery from LLO- versus control-liposomes. Data (mean ± SE), taken from Table 4.1, show the relative fluorescence levels in the cytoplasm of MTSV1-7 and ce2 cells after incubation with either control- or LLO-liposomes for 1.5 hours and subsequent heating at either 37°C or 42°C. The right-hand set of bars in the graph shows that ce2 cells have 22 times more calcein fluorescence than MTSV1-7 after incubation with LLO-liposomes and subsequent heating to 42°C.

While calcein delivered to ce2 cells from control-liposomes at 42°C was predominantly trapped in endosomes (Figure 4.5A), delivery from LLO-liposomes resulted in a diffuse cytoplasmic fluorescence (Figure 4.5B). Delivery was also specific for Her-2 overexpressing cells. The LLO-liposomes at 42°C delivered over 22-fold more calcein to the cytoplasm of ce2 cells than to the cytoplasm of MTSV1-7 cells (Table 4.1 and Figure 4.5).

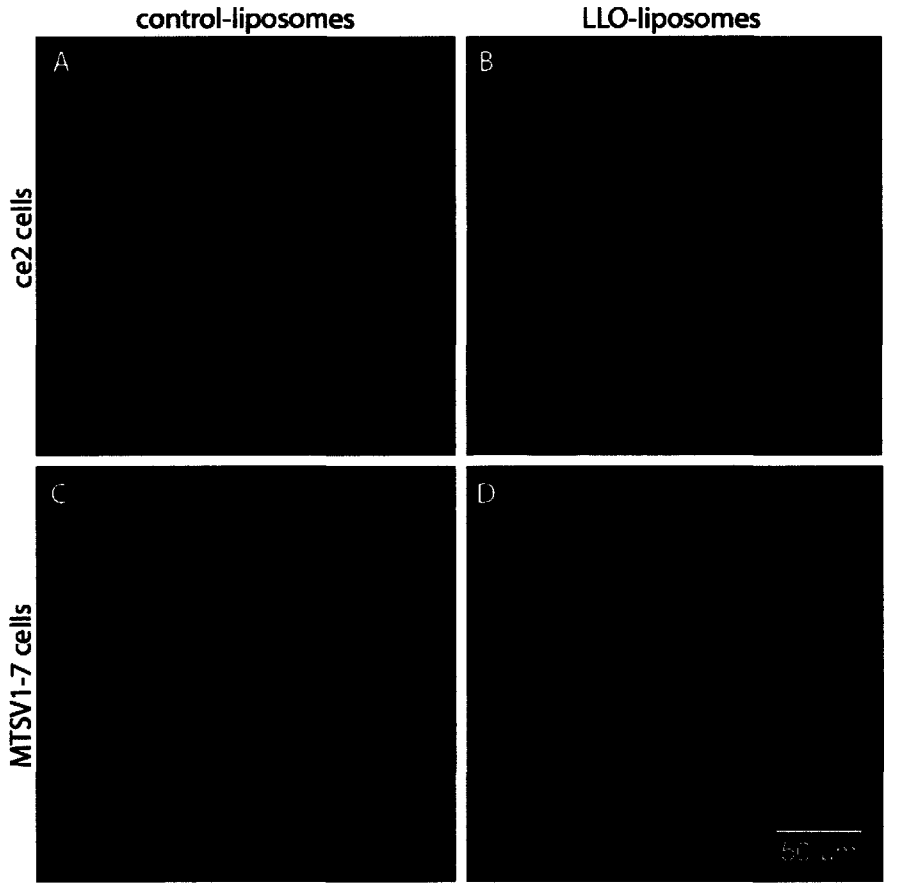

Figure 4.5. Delivery of calcein to ce2 cells versus MTSV1-7 cells at 42°C. All photographs show cells that have been exposed to liposomes for 1.5 hours at 37°C and subsequently heated to 42°C. Panels A and B show the delivery of calcein to ce2 cells incubated with control-liposomes (A) or LLO-liposomes (B). Similarly, C and D show MTSV1-7 cells incubated with control-liposomes (C) or LLO-liposomes (D). Images were taken at a magnification of 200x.

4.4.3 *Non-thermosensitive delivery from LLO-liposomes*

In our previous study (Kullberg et al. 2009), we noted that serum had the ability to destabilize the thermosensitive liposomes and cause significant leakage after 3 or 4 hours of incubation at 37°C (Kullberg et al. 2009). To test for the possibility of non-thermosensitive release in our present study, cytoplasmic delivery was measured after 1.5 hours, 4 hours and 10 hours incubation at 37°C.

Table 4.2. Cytoplasmic delivery of calcein over an extended incubation at 37°C.*

	1.5 Hours	**4 Hours**	**10 Hours**
MTSV1-7 control-liposomes	2.03 ± .14 (n = 169)	2.06 ± .14 (n = 171)	3.17 ± .17 (n =140)
Ce2 control-liposomes	4.21 ± .36 (n = 160)	3.72 ± .24 (n = 146)	6.51 ± .63 (n = 171)
MTSV1-7 LLO-liposomes	7.62 ± .44 (n = 148)	7.44 ± .48 (n = 153)	10.43 ± .71 (n = 163)
Ce2 LLO-liposomes	15.49 ± .65 (n = 172)	45.21 ± 1.82 (n = 164)	76.65 ± 3.1 (n = 168)

*Data (mean ± SE) represent relative fluorescence intensity of cytoplasmic calcein per cell. The number of cells analyzed (n) is indicated for each experimental condition. Cells were exposed to liposomes at 37°C for 1.5 hours and incubated for a total time of either 1.5, 4 or 10 hours at 37°C. Data are also represented graphically to show trends in cytoplasmic delivery over time (see Figure 6).

At 1.5 hours, the amount of calcein delivery to the cytoplasm of both ce2 and MTSV1-7 cells was relatively minor (Table 4.2 and Figure 4.6), but at 4 hours and 10 hours there were increasing amounts of fluorescent calcein being delivered to the cytoplasm of the ce2 cells treated with LLO-liposomes (Figures 4.6 and 4.7).

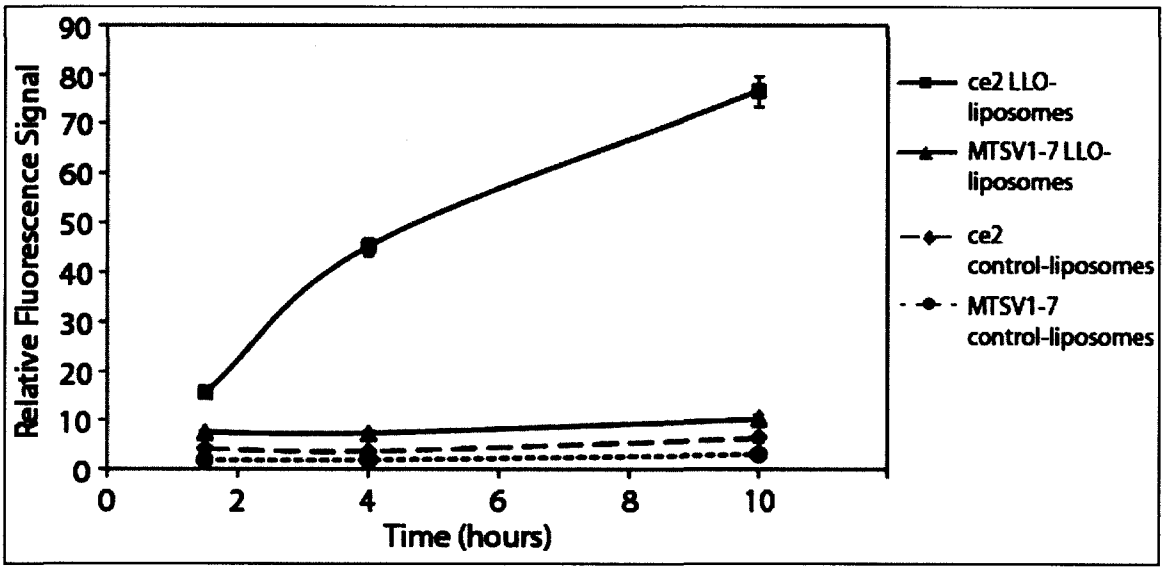

Figure 4.6. Cytoplasmic delivery of calcein, independent of hyperthermia. Data (mean ± SE), taken from Table II show the trend in cytoplasmic delivery for ce2 and MTSV1-7 cells treated with control-liposomes or LLO-liposomes over an extended incubation time at 37°C. MTSV1-7 cells incubated with control-liposomes (circles), MTSV1-7 cells incubated with LLO-liposomes (triangles) and ce2 cells incubated with control-liposomes (diamonds) have relatively minor increases in cytoplasmic delivery over the 10 hour period. Ce2 cells incubated with LLO-liposomes (squares) have a 5-fold increase in cytoplasmic delivery from 1.5 to 10 hours.

At the 10 hour time point, ce2 cells treated with LLO-liposomes had a 12-fold greater concentration of cytoplasmic calcein than when treated with control-liposomes (Table 4.2). In addition, the ce2 cells had a cytoplasmic calcein concentration that was over 7-fold greater than that in the MTSV1-7 cells at this same time point. The concentration of calcein in the non-heated ce2 cells at 10 hours was 82% of the concentration in ce2 cells heated to 42°C at 1.5 hours (Tables 4.1 and 4.2). The delivery of liposomal contents without heating presents an obstacle for exclusive heat targeted delivery to the tumor site, but represents an opportunity for a systemic treatment of metastasized Her-2 overexpressing cancer cells independent of hyperthermia.

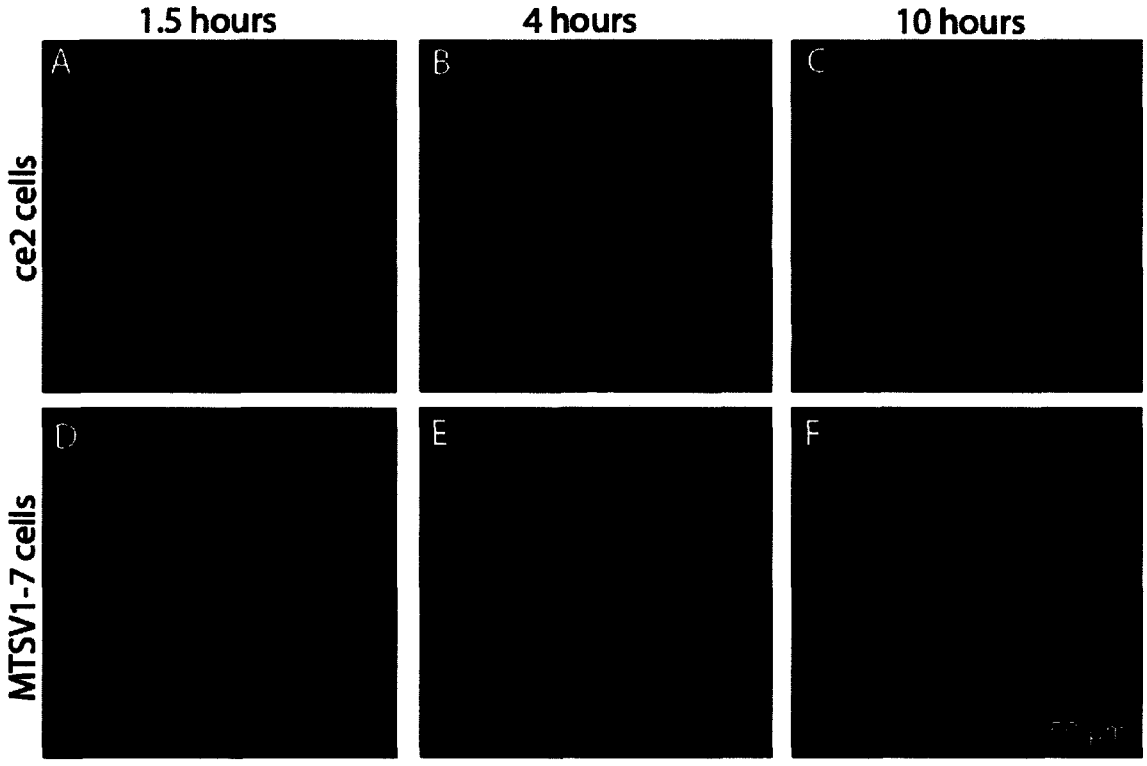

Figure 4.7. Delivery of calcein from LLO-liposomes to the cytoplasm after extended incubation at 37°C. A, B and C show representative photographs of cytoplasmic delivery to ce2 cells after a 1.5 hour incubation with LLO-liposomes and a total incubation time of 1.5 hr (A), 4 hr (B) or 10 hr (C) at 37°C. Likewise, D, E and F show MTSV1-7 cells exposed to the same conditions at 1.5 hr (D), 4 hr (E) or 10 hr (F). Analysis of the data indicates that at 10 hours, ce2 cells have a cytoplasmic calcein concentration that is over 7-fold higher than that in MTSV1-7 cells. Images were taken at a magnification of 200x.

An observation worth noting was that the addition of LLO to the liposomes increased the amount of liposomes bound to the plasma membrane of both ce2 and MTSV1-7 cells at 37°C. Cells exposed to control-liposomes showed little membrane binding (data not shown), while cells exposed to the LLO-liposomes had a speckled appearance all over their membrane (Figure 4.7D). Heating to 42°C caused the non-specifically bound liposomes to release their calcein into the extracellular medium, reducing the speckling and giving the cells a much darker appearance (Figure 4.5D). Analysis showed that the

measured calcein signal actually decreased when the MTSV1-7 cells were heated to 42°C (Table 4.1), most likely accounted for by the disruption of membrane-bound liposomes.

4.5 Discussion

Targeted drug delivery exploits unique properties of cancer cells for the purpose of increased specificity of chemotherapeutic treatment. Targeted liposomes are one of a set of rapidly emerging nanotechnologies, including polymeric nanovectors (Douziech-Eyrolles et al. 2007, Kommareddy et al. 2005), lipoprotein particles (Glickson et al. 2009), and dendritic nanocarriers (Xu et al. 2009, Bai et al. 2006), all aimed at specific delivery of therapeutic agents to cancer cells. In a previous study we combined thermosensitive liposomes, with trastuzumab, an antibody that targets the Her-2 receptor (Kullberg et al. 2009). In this study our goal was to enhance delivery of liposomal contents from the endosomal compartments to the cytoplasm of targeted cells by binding the liposomes to LLO, a protein that utilizes cholesterol to form pores in the endosomal membrane.

Lee et al. (1996) encapsulated LLO within pH sensitive liposomes and successfully increased delivery of macromolecules to the cell cytoplasm. By modifying this technique so that LLO was attached to the lipid bilayer of Her-2 targeted liposomes, we achieved a similar increase in delivery to the cytoplasm. LLO protein binding to the outside of the liposomes seems to have little effect on either liposome size or heat triggered release from the liposomes (Figure 4.3). However, we noted that LLO-liposomes attach to the plasma membranes of both cell lines much more readily than do control-liposomes (Figure 4.7). The stickiness of the LLO-liposomes may result in a reduction of both circulation time and concentration of the liposomes *in vivo*. This apparently non-specific membrane interaction could possibly be reduced by the inclusion of a longer PEG chain in the liposomal membrane so that the ability of LLO to form a bridge between the liposome and the cell would be restricted.

Our results show that the binding of LLO to the liposomes greatly increases cytoplasmic delivery to Her-2 overexpressing cells and that the delivery is dependent upon mild hyperthermia (Table 4.1 and Figure 4.4). Current chemotherapies lack the specificity needed to effectively treat and eliminate breast tumors. The strategy presented here offers the possibility of selective drug delivery to Her-2 overexpressing cancer cells while restricting delivery of drug to the tumor site by localized hyperthermia. In addition, treating tumors with hyperthermia has the benefits of increasing blood flow to the tumor, sensitizing the cancer cells to chemotherapies, and augmenting the immune response (Kong et al. 2001, Stahl et al. 2009). These therapeutic effects of hyperthermia could be synergistic with the targeting system described here.

For a heat-targeting strategy to be effective, liposomes should only deliver drug in response to hyperthermia and not at normal body temperature. However, we have found that after an extended incubation at 37°C, LLO-liposomes eventually deliver calcein to the cytoplasm of Her-2 overexpressing cells even without heating (Table 4.2 and Figure 4.6). This delivery creates a potential problem since non-malignant cells throughout the body that express low levels of Her-2 receptor may eventually experience cytoplasmic delivery of drug encapsulated in LLO-liposomes. Increasing specificity for the Her-2 receptor even further would help to limit undesired delivery to non-cancerous cells. In this regard, we have previously shown the possible benefit of a two-component strategy which would require the interaction of two Her-2 targeting liposomes within a single endosome for successful drug delivery to the cytoplasm of target cells (Kullberg et al. 2009). The probability of two liposomes overlapping in the same endosome is exponentially enhanced with increasing levels of Her-2 in the plasma membrane. By separating the LLO into one set of liposomes and the drug being delivered into another set, it would not be difficult to develop a two-component approach that would reduce drug delivery to cells expressing normal levels of Her-2.

4.6 Conclusions

We find that the addition of LLO to Her-2 targeted, thermosensitive liposomes markedly increases cytoplasmic delivery of liposomal contents to targeted cells. The intrinsic ability of LLO to bind to liposomes makes coupling of the protein straightforward and causes little change in the original characteristics of the liposomes. After being endocytosed, LLO compromises the endosomal barrier and enables effective delivery of liposomal contents to the cytoplasm. Thus, the addition of LLO to preformed liposomes has the potential to greatly enhance the effectiveness of targeted liposome therapies.

4.7 Acknowledgements

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4.8 Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Chapter 5: Targeting Her-2 Overexpressing Cells with Gelonin Filled Liposomes[1]

5.1 Abstract

A preliminary experiment was carried out to test the cytotoxic effect of targeted gelonin delivery on mammary epithelial cells that overexpress the human epidermal growth factor receptor 2 (Her-2). To deliver the cytotoxin, gelonin, to Her-2 overexpressing cells, we have utilized liposomes that are conjugated to the Her-2 targeting antibody, trastuzumab. These liposomes are also attached to listeriolysin O (LLO), a protein that forms pores in cholesterol containing membranes. After liposomes bind and internalize into Her-2 overexpressing cells, LLO molecules form pores in the endosomal membrane, enabling cargo that has leaked from the liposomes direct access to the cellular cytoplasm. In a previous publication, we demonstrated selective delivery of a fluorescent marker, calcein, to the cytoplasm of Her-2 overexpressing cells with minimal delivery to normal cells. In this work, the calcein was replaced with a potent plant toxin, gelonin, and the ability of the system to kill Her-2 overexpressing mammary cells was tested *in vitro*. The results of this experiment suggest that gelonin delivery by targeted liposomes killed 90% of Her-2 overexpressing cells (ce2 cells) over three days, and killed about 40% of cells expressing a lower level (10%) of Her-2 receptors. While the specificity of killing needs to be increased, this preliminary result encourages further investigation of targeted killing of breast cancer cells using Her-2 conjugated liposomes.

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[1] Kullberg M, Owens JL, Mann K. 2010. Targeting Her-2 Overexpressing Cells with Gelonin Filled Liposomes. The data presented in this chapter is preliminary.

5.2 Introduction

Delivery systems that can penetrate the endosomal membrane and deliver drug directly to the cellular cytoplasm are of growing importance as the number of new therapeutic macromolecules increases (El-Sayed et al. 2009). Currently, RNAi, gene therapy and cytotoxic peptides offer sophisticated alternatives to the traditional small molecule chemotherapy, but their therapeutic benefits will remain unrealized without an effective means of delivery to the cytoplasm (Belting et al. 2005). These chemotherapeutic macromolecules are commonly targeted to the cellular endocytotic system either through antibody conjugation or nanoparticle encapsulation. Encapsulating the therapeutic agents within liposomes that target cancer cells is an effective way of achieving large concentrations of the macromolecule within cellular endosomes (Amiji 2007). The liposomes protect the encapsulated package and preferentially localize in the tumor tissue due to the leaky vasculature common in almost all solid tumors (Torchilin 2000, Maeda et al. 2009). However, unless they are supplied with a method for endosomal escape, the delivered macromolecules will be exposed to the low pH environment of the endosome and eventually to the degradative enzymes in the lysosome (Fretz et al. 2007, Xu et al. 2008).

In a previous study, we developed a cytoplasmic delivery technique using a pore forming protein, LLO, for penetrating the cellular endosome (Lee et al. 1996, Provoda et al. 2003, Kullberg et al. in press). LLO is attached to the outside of the liposomes and after being endocytosed with the liposomes, LLO forms pores in the endosomal membrane through which macromolecules can travel to reach the cytoplasm. Using this method with Her-2 targeting liposomes, we were able to target a fluorescent marker to the cytoplasm of Her-2 overexpressing cells with high specificity (Kullberg et al. in press). In the current

study, we have replaced the encapsulated calcein with the toxin gelonin, a 30 kDa toxin isolated from the seeds of a plant, *Gelonium multiforum.*

Gelonin is ideal for this delivery system because it is incapable of escaping independently from a cellular endosome and is relatively non-toxic to cells, with an LD50 of 40-75 mg/kg in mice (Provoda et al. 2003, Dietze et al. 2006). However, when gelonin and LLO co-locate in an endosome, LLO forms pores in the endosomal membrane, allowing gelonin access to the cytoplasm where it is an extremely potent toxin. Provoda *et al.* (2003) co-encapsulated LLO and gelonin into a single set of pH-sensitive liposomes and allowed cells to engulf these liposomes. When the LLO and gelonin were released from the liposome into an endosome, gelonin traveled through LLO formed pores into the cytoplasm where it inhibited ribosome activity, resulting in cell death. Using gelonin filled liposomes, we have tested the ability of our trastuzumab-LLO-liposomes to specifically inhibit the growth of Her-2 overexpressing cells *in vitro.*

The results reported here are preliminary. Although data analysis was performed on three images, these images were taken from a single experiment and must be repeated to confirm the results.

5.3 Materials and Methods

5.3.1 *Materials*

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (MPPC) and 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DPPG) were purchased from Avanti Polar Lipids (Alabaster, USA). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[poly(ethylene glycol)-3400]-N-hydroxy succinamide (DSPE-PEG(3400)-NHS) was purchased from Shearwater Polymers (Huntsville, USA), now Nektar Therapeutics (San Carlos, USA). Gelonin was purchased from Enzo Life Sciences (Plymouth, USA). Dulbecco's phosphate-buffered saline

(DPBS) was bought from Gibco BRL (Gaithersburg, USA). CL-4B Sepharose gel was purchased from Amersham Biosciences (Uppsala, Sweden). Trastuzumab was a kind donation from Dr. Max Rabinowitz at Alaska Oncology and Hematology, Anchorage, AK. LLO-pEt29-DP-E3570 transfected E. coli were provided by Dr. Dan Portnoy at University of California, Berkeley, CA. All other chemicals and reagents were purchased from Sigma Chemicals Company (USA).

5.3.2 *Cell culture*

The cell lines MTSV1-7 and ce2 were generously supplied by Dr. Joyce Taylor-Papadimitriou at the Breast Cancer Biology Group, King's College London School of Medicine, U.K. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum and supplements as previously described (Kullberg et al. 2009).

5.3.3 *Preparation of liposomes filled with gelonin*

Liposomes were made with lipids DPPC:MPPC:DPPG:DSPE-PEG(3400)-NHS at a molar ratio of 82:10:3.5:4 and prepared by the film hydration-extrusion method (Torchilin and Weissig 2003). The procedure for conjugating liposomes to trastuzumab and filtering to 100 nm or 200 nm was described in chapter 3 of this document. Liposomes were filled with gelonin by mixing 50 µl of liposomes (0.5 mg lipid/ml) with 50 µl of a 50 mM gelonin solution in gelonin storage buffer (Enzo Life Sciences). The liposomes were heated to 42°C for 3 min, allowing gelonin to flow into the liposomes which are leaky at the elevated temperature. After cooling, the liposomes were separated from unencapsulated gelonin by column chromatography on a CL-4B column.

5.3.4 *Conjugation of LLO to liposomes*

LLO was conjugated to both 200 nm and 100 nm liposomes. LLO was purified as described in chapter 4. An aliquot of 0.4 ml of CL-4B purified gelonin filled liposomes (0.33mg lipid/ml) was mixed with an 8µl aliquot of LLO (2.0 mg/ml in storage buffer). The mixture was left at room temperature for 10 min and then 8 µl of cholesterol (25

mg/ml in 200-proof ethanol) was added to the solution. After rotating slowly for 30 min at room temperature, the cholesterol was pelleted by centrifuging at 3800 g for 3 min. The supernatant containing the gelonin filled liposomes (gel-LLO-liposomes) was removed and kept at 4°C until used. Also, a control set of liposomes was prepared without gelonin, keeping all other parameters the same (LLO-liposomes)

5.3.5 *In vitro delivery from gelonin filled liposomes*

8-well chamber slides (Nalge Nunc International, Rochester, USA) were used for cell culture studies, with the ce2 and MTSV1-7 cells grown in 0.4 ml of DMEM medium/well. Two concentrations (differing by 2-fold) of 100 nm liposomes were tested. For the low liposome concentration sample, the medium was removed 24 to 48 hours after subculture and replaced with a solution comprising 0.15 ml of complete medium and 0.05 ml of either gel-LLO-liposomes or LLO-liposomes in DPBS. The higher concentration consisted of 0.10 ml of complete media and 0.10 ml of either gel-LLO-liposomes or LLO-liposomes The final lipid concentration in the high concentration condition was 0.166 mg/ml and in the low concentration condition was 0.083 mg/ml. After incubating the cells with liposomes for 1.5 hrs at 37°C and 5% CO_2, the liposome containing media was removed and replaced with liposome-free medium. For the unheated experiment, slides were then put back in the incubator at 37°C and 5% CO_2. For the heated delivery experiment, the slide chamber was submerged in a water bath at 42°C for four minutes and then placed back in the incubator. For both unheated and heated experiments, the cells were left for 3 days to allow any cytoplasmic gelonin time to affect the cells. After the 3 day incubation, the medium was removed and replaced with a 0.2 mM calcein AM solution for the purpose of measuring cell viability. After 10 min incubation at 37°C, the cells were fixed with 4% paraformaldahyde and imaged using fluorescence microscopy.

5.3.6 *Quantification of viability*

Viability was determined by gathering 3 image fields from random locations for each experimental condition, using a low powered 50x magnification to incorporate as many cells as possible into each image. Background readings were taken from three locations on the slide, averaged and subtracted from the total mean fluorescence to give a mean fluorescence reading for the plate. This reading is proportional to the number of viable cells that are located within the image field.

5.4 Results

5.4.1 *Viability of cells exposed to gelonin liposomes*

Gelonin filled liposomes of 100 nm and 200 nm were tested for their cytotoxic effects on the mammary epithelial cell lines, MTSV1-7 and ce2. The cell lines are identical except that the ce2 cells have been transfected with Her-2 DNA so that they overexpress the receptor by 10-fold. When incubated with gelonin filled liposomes, the ce2 cells are significantly damaged, showing a much sparser cell population and lower cell viability readings than ce2 cells incubated with gelonin-free control LLO-liposomes (Table 5.1, Figure 5.1). In all conditions tested, including 200 nm liposomes, 100 nm liposomes, high and low concentrations of liposomes, and heating to 42°C, gel-LLO-liposomes killed about 80 to 90% of ce2 cells over a period of three days.

MTSV1-7 cells are also damaged by the gel-LLO-liposome experimental conditions (Table 5.1). The data suggest that the best killing specificity for ce2 cells was obtained by the lowest dose of gelonin, as if the killing reactions were approaching saturation in the ce2 cells. The least damage of MTSV1-7 cells was obtained by a low, unheated dose of 100 nm liposomes, killing about 40% of cells after 3 days. Anything that may have increased the dose of gelonin – higher liposome concentration, larger liposomes, or heating – increased the killing of MTSV1-7 cells (Table 5.1)

Table 5.1. Percentage death of ce2 and MTSV1-7 cells treated with gelonin filled liposomes.*

Liposome diameter (nm)	Liposome concentration	Heated to 42°C or 37°C	% of ce2 cells killed	% of MTSV1-7 cells killed
100	low	37°C	88 ± 5	39 ± 3
100	low	42°C†	89 ± 2	49 ± 6
200	low	37°C	88 ± 3	71 ± 1
100	High	37°C	77 ± 8	72 ± 8

*To determine viability, cells were treated with calcein-AM, producing a cell fluorescence that is proportional to cell viability. Estimates of cell killing were obtained by first calculating percentage cell survival, which is the ratio (multiplied by 100) of fluorescence intensity of cells treated with gelonin filled LLO-liposomes to that of cells treated with control LLO-liposomes that contain no gelonin. Subtracting the percentage cell survival from 100 gives the percentage of cells killed. Data are presented as mean ± SE, where n=3. Liposomes were filtered to either 200 nm or 100 nm and then added to the cells at a low or high concentration with lipid concentrations of .083 and .0166 mg/ml respectively. Cells were incubated with the liposomes for 1.5 hours at 37°C and 5% CO_2 and then incubated in fresh media for 3 days at 37°C

† After incubating for 1. 5 hours at 37°C, the cells shown in the second row of the table were heated to 42°C for four minutes to trigger heat release of gelonin from the liposomes.

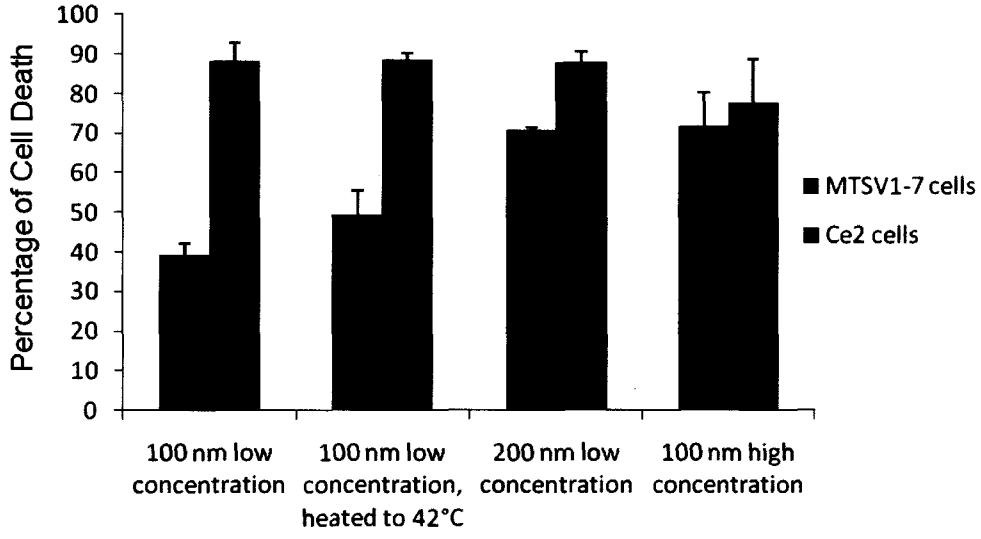

Figure 5.1. Percentage death of cells after exposure to gelonin filled liposomes. The ce2 cells are significantly damaged by treatment with gel-LLO-liposomes in all the various experimental conditions. MTSV1-7 cells are also damaged in each condition, but there is less damage with the smaller 100 nm liposomes administered at the low concentration. The specificity of cytotoxicity for ce2 cells was somewhat preserved even when the cells were heated to 42°C.

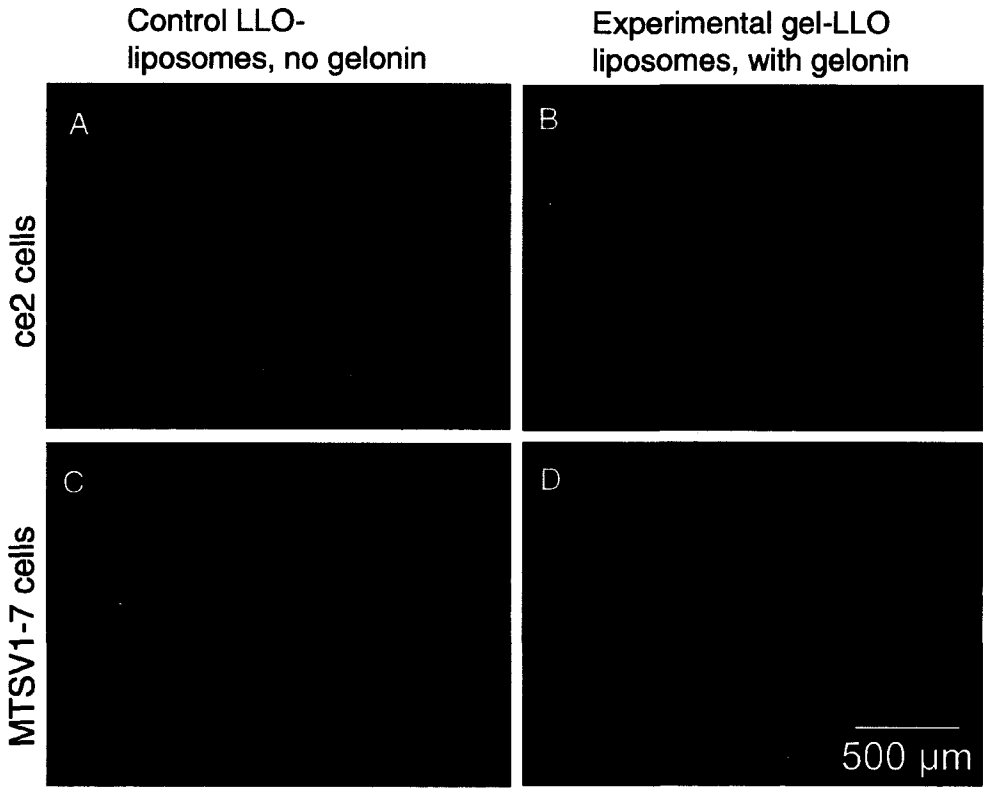

Figure 5.2. MTSV1-7 and ce2 cells treated with 100 nm gel-LLO liposomes at low concentration. The low concentration of 100 nm gel-LLO liposomes caused significant damage to ce2 cells (B), while ce2 cells treated with control LLO-liposomes remain unharmed (A). MTSV1-7 cells treated with the gel-LLO-liposomes (D) are also damaged slightly by the treatment compared to control MTSV1-7 cells. Images were taken at a magnification of 50x.

5.5 Discussion

Methods for delivering more potent and sophisticated chemotherapeutic molecules must be developed to effectively treat Her-2 overexpressing breast cancers. Nanoparticles which can be functionalized and tailored to specifically target large quantities of macromolecules to target cells offer an alternative to traditional small molecule chemotherapies. The Her-2 targeting liposomes described in this study are attached to

the pore forming protein LLO which aids in the cytoplasmic delivery of the encapsulated protein gelonin, an efficient ribosome inactivating toxin.

The preliminary results indicate that 100 nm gelonin filled liposomes are effective in specifically targeting Her-2 overexpressing cells when administered in low concentration. At the low concentration of liposomes, Her-2 overexpressing ce2 cells receive enough of the gelonin to be significantly damaged while the lower internalization rates of the normal MTSV1-7 cells result in lower cytotoxicity. With a higher concentration of liposomes or larger diameter liposomes, the MTSV1-7 cells with a normal level of Her-2 expression also internalize cytotoxic amounts of gelonin, resulting in a decreased specificity for the Her-2 overexpressing cells. Determining the concentration of liposomes that kills the Her-2 overexpressing cells while leaving the normal cells unharmed is a parameter that must be maximized.

The liposomes used in these experiments are thermosensitive and were originally designed with the intention of locally heating tumor tissue to trigger the release of drug from endosome into the tumor cell cytoplasm. However, in a previous experiment, we noted that even without heating, the liposomes were eventually degraded within the cell, resulting in leakage of an encapsulated fluorescent marker and delivery to the cytoplasm. A similar effect seems to be occurring even when the fluorescent marker is replaced by the toxin gelonin. We hypothesized that the breakdown of the liposome was occurring in lysosomes and that the gelonin released into the lysosomes would be rendered ineffective due to the degradative conditions. This is not the case as the cytotoxic effect of the gelonin containing liposomes seems similar whether the cells are heated or left at 37°C. These results show that a heated delivery approach using this system may not increase the efficacy of drug delivery. The inability to trigger release with hyperthermia would reduce the anatomical specificity of the delivery system for treatment of localized tumors, but would not effect the systemic treatment of metastasized tumors, where localized heating would not be applicable.

While gelonin encapsulated liposomes appear to be effective in specifically damaging Her-2 overexpressing cells, it is important to characterize this delivery system with other chemotherapeutic agents. Gelonin is a relatively large therapeutic molecule and is therefore difficult to encapsulate using our method of heating the liposome, which causes a temporary permeability and inflow of drug. In order to encapsulate gelonin, we needed to use a high osmolarity buffer, which could compromise the integrity of the liposomes. In the past, high osmolarity solutions have resulted in an unspecific delivery of calcein to the cytoplasm (data not shown), which could also be occurring here with the gelonin. A smaller chemotherapeutic agent that might work well with this drug delivery system is bleomycin, which has a molecular weight of 1,415 g/mol. Bleomycin functions by creating single- and double-strand breaks in DNA, resulting in cell death (Hecht 2000). One benefit of bleomycin is that the drug is already used as a chemotherapeutic agent in humans, so its side effects and tolerable concentrations have been well characterized. The drug has a limited ability to cross plasma membranes and considerable study has been attempted to increase its cytoplasmic concentration within target cells (Hofmann et al. 1999). Since bleomycin cannot escape from endosomes readily, a liposome system consisting of LLO and bleomycin should be effective in enhancing cytoplasmic concentrations of the drug.

The preliminary results in this study show that 100 nm liposomes which encapsulate gelonin specifically target and kill Her-2 overexpressing cells *in vitro*. The ability to target macromolecules to the cytoplasm of target cells is an elusive goal which must be achieved to utilize the many chemotherapeutic macromolecules currently available. While this system was tested with gelonin, the toxin could be substituted with RNAi, DNA, chemotherapeutic molecules or other proteins. Attaching LLO to the outside of targeted liposomes represents a new method for delivery of macromolecules to the cytoplasm and could help to improve the efficacy of delivery from cargo-laden liposomes.

5.6 Acknowledgements

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5.7 Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Chapter 6: Conclusions

6.1 Conclusions

The aim of this doctoral project was to overcome some of the obstacles that hinder liposomal based drug delivery to Her-2 overexpressing breast tumors. The obstacles of drug delivery include avoiding the immune system, penetrating deep into tumor tissue, specifically targeting the cancer cells and delivering drug directly into the cellular cytoplasm. This work focuses on the barriers to drug delivery that are present once liposomes come into contact with the tumor cells. The goal of the research was to make a liposome system that was very specific for Her-2 overexpressing cells and to develop a system that could deliver macromolecules directly to the cytoplasm of these cells.

Specificity for Her-2 overexpressing cells was achieved by creating thermosensitive liposomes conjugated to the antibody trastuzumab and utilizing these liposomes in a new two-component targeting approach (Kullberg et al. 2009). The technologies of thermosensitive liposomes and Her-2 targeting liposomes were combined by our group and another group, working simultaneously but independently (Puri et al. 2008, Kullberg et al. 2009). Our results show the first *in vitro* study of these liposomes, displaying their ability to bind and internalize into Her-2 overexpressing cells with high specificity. After exposure to a heat source, the liposomes released their encapsulated cargo into the cellular endosomes. The two-component therapy proposed in this study shows that requiring the overlap of two separate liposomes in an endosome for effective drug delivery could significantly increase specificity for Her-2 overexpressing cells. Since normal cells throughout the body express basal levels of Her-2, this method could be important for enhancing the specific targeting of tumor cells.

To deliver macromolecules to the cytoplasm of target cells, we modified a technique which encapsulated LLO, a pore-forming protein that forms pores in cholesterol containing membranes. Instead of encapsulating LLO within liposomes, the protein was attached to the outside of the Her-2 targeting liposomes. After the liposomes were internalized into Her-2 overexpressing cells, LLO formed pores in the endosomal membrane, enabling delivery of liposomal cargo to the cellular cytoplasm (Kullberg et al. in press). When gelonin was encapsulated within these liposomes, the drug delivery system displayed specific cytotoxicity for Her-2 overexpressing cells. Methods for delivering macromolecules to the cytoplasm are limited and of vital importance (El-Sayed et al. 2009). The development of this technique with LLO offers a new method for facilitating endosomal escape and can be easily incorporated into targeted liposome delivery systems.

While this liposome delivery system successfully targeted Her-2 overexpressing cells *in vitro*, there is still a large leap to *in vivo* effectiveness. One of the greatest difficulties for nanoparticle delivery systems is penetrating deep into the tumor tissue (Lunt et al. 2008). Even while efficient extravasation into tumor tissue occurs through enhanced penetration and retention (EPR), travel of the large particles within the tumor interstitium can be limited. For this reason, a two-component therapy employing gelonin laden liposomes has not been pursued in this research project. While the two-component therapy increases specificity for Her-2 overexpressing cells *in vitro*, it requires overlap of two species of liposomes within the cellular endosomes, and that in turn requires a larger concentration of liposomes at the cell surface. Because reaching each tumor cell is such a barrier to treating tumors, it seemed important to first concentrate on delivery that necessitated only a single potent component. Gelonin has been shown to kill cells when as few as 6 to 10 molecules reach the cytoplasm (Eiklid et al. 1980). With the one-component targeting technique described in this research, which allows for delivery of liposomal contents directly to the cellular cytoplasm, it should be possible to kill a cell with a single gelonin filled liposome. Many of the liposomal systems being tested in

clinical trials release a large amount of a small chemotherapeutic agent in the vicinity of the tumor (Hauk et al. 2006, Amiji 2007). That chemotherapeutic agent travels through the tumor tissue, but also through the normal tissue, exerting cytotoxic effects that limit the therapeutic dose. By creating highly specific liposomes that deliver a very potent package directly to the cytoplasm of Her-2 overexpressing cells, it should be possible to treat Her-2 overexpressing breast tumors more effectively.

The first step in the transition between *in vitro* and *in vivo* studies will be to carry out a biodistribution study in a mouse model. Every liposome system behaves differently within the body, and in particular LLO attached to the outside of the liposome may affect the *in vivo* characteristics of the system. While PEG should be protecting the liposomes from the immune system, it is alarming that the LLO liposomes stick non-specifically to both ce2 and MTSV1-7 cells. This stickiness could result in enhanced recognition by the immune system and increased rates of liposome clearance. Also of importance is seeing how the liposomes travel through the tumor interstitium and estimating the number of liposomes that are internalized into cells throughout the tumor. Fluorescence microscopy experiments can give much information on the biodistribution and location of liposomes within the body, tissues, and cells (Lajavardi et al. 2007).

Current chemotherapeutic approaches are limited by their lack of specificity for the tumor cells, resulting in adverse side effects and ineffective treatment. The recent emergence of nanotechnology combined with exciting new chemotherapeutic RNAi, DNA and proteins has given new hope. By attacking the barriers preventing effective delivery from these particles one at a time, scientists are steadily advancing the field of nanotechnology, moving toward more effective chemotherapies. By combining and modifying some of the techniques developed by others, this thesis research has hopefully moved the field of nanotechnology a small step further.

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